TABLE II. Amount of Plasticizer Eluted from PVC Sheet Incubated in MAP/RCC at 37°C During 10 Weeks Under the Conditions of Continuously Gentle Shaking.

Plasticizer	Final content (w/w %)	Elution amount (μg/ml)										
		6 days	13 days	21 days	27 days	35 days	40 days	49 days	56 days	63 days	70 days[a]
TOTM	44.0	0.04±0.01	0.04±0.01	0.05±0.01	0.06±0.01	0.11±0.07	0.25±0.19	0.35±0.34	0.12±0.05	0.09±0.04	0.27±0.09
DEHP	33.7	15.0±2.60	16.2±3.59	28.3±6.78	28.5±6.06	34.7±4.54	38.8±5.57	42.4±6.22	40.1±11.5	45.0±7.86	53.1±6.07
DINCH	50.5	3.73±073	4.34±1.32	11.3±1.20	12.3±2.38	16.3±4.91	20.4±2.00	22.5±4.24	25.0±3.77	27.8±5.33	36.5±4.34
	44.0	3.55±0.42	5.27±1.15	9.85±1.34	11.9±2.81	15.7±3.91	19.5±2.11	21.3±3.65	24.8±3.93	29.2±2.98	32.5±4.64
	35.7	3.31±0.66	5.03±1.59	10.2±2.17	10.7±3.19	13.5±2.31	18.3±4.87	18.2±2.02	22.0±2.82	25.2±3.52	33.3±2.39
	24.5	2.88±0.51	4.40±1.13	9.34±1.95	9.92±3.55	14.2±2.91	17.3±3.20	15.5±2.67	18.9±2.48	21.3±2.09	26.1±4.79
DIDP	50.5	0.62±0.08	0.92±0.34	1.87±0.23	1.81±0.45	2.94±0.73	3.70±0.51	3.74±0.65	4.00±0.72	5.02±0.54	5.96±0.66
	44.0	0.54±0.08	1.17±0.31	1.43±0.39	1.63±0.60	2.37±0.31	3.60±0.84	4.25±0.46	4.33±0.89	4.53±1.45	5.51±0.56
	35.7	0.53±0.05	0.86±0.20	1.57±0.20	1.67±0.55	2.41±0.63	3.39±0.52	4.05±0.48	3.75±0.34	4.40±0.88	5.35±1.39
	24.5	0.45±0.05	0.92±0.31	1.40±0.22	1.59±0.45	2.32±0.40	3.34±0.60	3.71±0.60	3.31±0.25	4.57±0.83	4.80±0.75
DOTP	50.5	40.8±4.67	45.6±14.7	58.4±21.1	83.0±21.9	92.5±23.6	110±15.1	116±12.8	123±12.3	128±26.0	150±26.0
	44.0	42.3±5.80	52.9±11.8	62.1±22.6	86.2±15.7	91.9±13.9	91.0±11.1	129±21.7	122±18.9	128±16.8	143±16.8
	35.7	28.8±7.55	36.0±12.1	53.4±15.7	71.3±18.7	73.2±12.9	97.0±23.1	112±21.2	101±11.6	94.6±16.6	117±8.00
	24.5	24.1±3.63	29.8±1.70	44.2±2.14	56.1±4.48	61.3±9.35	70.2±12.5	75.9±6.08	71.6±17.4	97.0±36.8	78.4±13.7
Control (DEHP)	–	tr[c]	tr	tr	tr	tr	tr	tr	tr	tr	tr

[c]tr, trace amount less than LOQ.
[a]Significant difference ($p < 0.01$) was detected between the elution amounts of DEHP and other plasticizers.

the plasticizer molecules may be related to differences in the elution behavior, depending on the type and number of the carboxy-ester groups present in the plasticizer molecule.

The type of anticoagulant and the viability of RBCs may be closely correlated with the expression of the hemolysis suppression effect of the plasticizer to RBCs.[25] The biochemical property of heparinized blood is very different from that of MAP/RCC, and hence, it is meaningful that the effect of the plasticizer on these two blood types is compared to evaluate the difference in the anticoagulant activity and the viability of RBCs as a typical case. DEHP, DIDP, DINCH, and DOTP exhibited a hemolysis suppression effect against MAP/RCC, but not against heparinized blood. Heparin inhibits the coagulation system by activating antithrombin, and the citrate present in CPD and MAP solutions inhibits the coagulation cascade by binding to Ca^{2+} ions (Factor IV). MAP solution has higher ability than heparin with respect to preserving the viability of RBCs because of the presence of glucose and mannitol in the solution. In fact, the lifetime of RBCs in heparinized blood is approximately half that of MAP/RCC, suggesting that the decrease in RBC viability may dominate over the stabilization of the cell membrane by these plasticizers. Probably, the ATP present in the heparinized blood was nearly depleted after 21 days, corresponding to the allowed limit of storage for human blood collected in heparin.

Recently, Hirata-Koizumi et al. reviewed the toxicity of alternative plasticizers such as DEHTP, DINCH, ATBC, diisononyl adipate (DINA), 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (TXIB), and tri-n-butyl citrate (TBC).[26] The overall toxicity of DOTP is still unknown. However, the results of the repeated dose toxicity, reproduction/development toxicity, bacterial reverse mutation, and chromosomal aberration tests conducted by BOZO Research Center (Shizuoka, Japan) as commissioned projects of the Japanese Ministry of Health, Labor, and Welfare showed that there is a high possibility that the plasticizer could be used to produce medical-grade PVC products because of minimal toxicity. These data are uploaded in the websites (http://dra4.nihs.go.jp/mhlw_data/home/pdf/PDF2915-49-3d.pdf, http://dra4.nihs.go.jp/mhlw_data/home/pdf/PDF2915-49-3e.pdf, and http://dra4.nihs.go.jp/mhlw_data/home/pdf/PDF2915-49-3f.pdf). DOTP is a cold-resistant plasticizer and has been used to produce a power-transmission line. Cold resistance of DOTP higher than that of DEHP may provide an advantage with respect to preventing cracking, which rarely occurs during cryopreservation of DEHP-PVC containers for blood products such as human plasma. The amount of DOTP eluted from the PVC sheet is relatively higher than that of DEHP, but that amount could be artificially adjusted to the minimal amount to suppress hemolysis (~10 μg/mL as described above) by deceasing the mixing ratio of DOTP to PVC and mixing precise doses of plasticizers with low leachability, such as DINCH or TOTM, to reduce patient exposure to these substances during medical treatments. The amount of plasticizers eluted from heat-pressed PVC sheets seems to be higher than that from T-die molded PVC sheets used to manufacture commercial PVC products. A possible reason

for this behavior could be the different orientation and density of the PVC molecules present in the sheets. In addition, the conditions used in this study are different from typical RBC storage because the blood was not leukocyte-reduced and was continuously in contact with the air present in the bottle head. On the basis of these results, preparation of a novel blood container prototype made from PVC sheets containing DOTP instead of DEHP and studies on the chemical, physicochemical, biological, and dynamical characteristics of the prototype are now in progress in our laboratory.

CONCLUSIONS

DINCH and DOTP are possible candidates as a replacement for DEHP in RBC storage bags because of their hemolysis suppression abilities. Although DIDP itself was effective in suppressing hemolysis, it could not be used as an alternative plasticizer because the amount of DIDP eluted from the PVC sheet did not reach the concentration essential for exhibiting its suppression effect.

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Ⅲ．研究成果の刊行に関する資料⑤

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Development and performance evaluation of a positive reference material for hemolysis testing

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Abstract: This study deals with the development and performance evaluation of a positive reference material for hemolysis testing, which is used for evaluating the biological safety of medical devices. Genapol X-080, a nonionic detergent, was selected as a candidate hemolytic substance in a survey of 23 chemical compounds; it showed significant hemolytic activity against rabbit defibrinated blood at concentrations more than 20 μg/mL. A polyvinyl chloride (PVC) sheet spiked with 0.6% (w/w) of the compound exhibited weak hemolytic activity in direct contact and/or extract-based assays after 4 h incubation at 37°C. A PVC sheet containing 5.8% (w/w) Genapol X-080 induced complete hemolysis in both assays. The amount of Genapol X-080 eluted from each PVC sheet during hemolysis testing using the direct contact method increased time-dependently and reached 25.6 (former sheet) or 1154 (later sheet) μg/mL after 4 h incubation, which was similar to or much higher than the critical micelle concentration, respectively. Similar elution behavior was observed using the extract-based method, and the Genapol X-080 content in test solutions prepared by autoclave extraction of both sheets was 22.5 and 358 μg/mL, respectively, indicating a clear relationship between the degree of hemolytic activity and the eluted amount of Genapol X-080. Thus, a PVC sheet spiked with a compound exhibiting different hemolytic activity depending on its concentration may be useful as a positive reference material to validate the hemolysis tests. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 102B: 1809–1816, 2014.

Key Words: hemolysis test, positive control, biological safety evaluation, guidance

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INTRODUCTION

Biological safety evaluation of raw materials or medical devices must be conducted using risk analysis techniques specified in ISO 14971.[1] The intended use or purpose and the safety properties of a medical device must be clarified or known foreseeable hazards must be identified, and the risk of each hazard must be anticipated. Items to be evaluated to determine the biological safety of each medical device are selected according to the requirements specified in ISO 10993-1.[2]

As a general rule, the items must be evaluated depending on their categorization in accordance with the nature of body contact and the duration of contact. Hemocompatibility testing is required to evaluate the safety of a medical device or material that has contact with blood.[4–7] Hemolysis testing is a part of hemocompatibility testing, which involves tests for thrombosis, coagulation, platelets, hematology, and the complement system. Mechanical and biochemical factors affect hemolysis. For medical devices such as membrane

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TABLE I. List of a Candidate of Hemolytic Compound and the Hemolytic Activity Estimated by Simple Hemolysis Test Method

Compound (manufacturer No.)[a]	Dissolved solvent	Final concentration for test (μM)	Hemolytic ratio (%) [d]
Triphenyl phosphate (1)	DMSO[b]	500	0.5
Lactic acid (1)	PBS	5	1.3
Orcinol anhydrous (1)	PBS	50	0.9
Lead acetate (1)	PBS	50	1.0
Sodium chlorate (1)	PBS	50	0.7
Diphenyl disulfide (2)	DMSO	500	93.0
Dimethylglyoxime (1)	DMSO	500	0.3
3-Phenylazo-2,6-diaminopyridine monohydrochloride (2)	DMSO	500	0.6
5-Aminosalicylic acid (1)	DMSO	500	0.4
4-Nitroaniline (3)	DMSO	500	0.7
o-Aminophenol (1)	DMSO	500	6.3
Aniline (1)	DMSO	500	0.4
p-Aminodipheylamine (1)	DMSO	500	0.3
2-Nitrophenylhydrazine hydrochloride (2)	DMSO	500	0.3
Sulfasalazine (2)	DMSO	500	1.4
Nitrobenzene (1)	DMSO	500	0.5
Resorcinol(1)	DMSO	500	1.2
Genapol X-080 (3)	DMSO	50[c]	100.9
2,4,6-Trinitrophenyl (1)	DMSO	500	0.3
Zineb standard (4)	DMSO	500	8.6
D-Penicillamine hydrochloride (5)	DMSO	500	0.6
1-Naphthylamine (3)	DMSO	500	0.4
p -Aminosalicylic acid sodium salt dihydrate (5)	DMSO	500	0.3

[a] (1) Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), (2) Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), (3) Sigma-Aldrich Co. (Tokyo, Japan), (4) Kanto Chemical Co. (Tokyo, Japan), and (5) MP Bio Japan K.K. (Tokyo, Japan).
[b] Compound dissolved in DMSO was diluted to 100 folds with PBS at the final concentration for hemolysis test.
[c] μg/mL.
[d] Hemolytic ratio after 4 h incubation.

oxygenators or hemodialyzers that may physically damage blood cells, the test should be designed considering mechanical factors, for example, using a blood circulation system. For devices that have little mechanical impact on blood cells, established static or semi-static *in vitro* testing can be used. This testing may be also useful for devices with mechanical impact on blood to evaluate the impact of materials on hemolysis.

Biological testing methods have to be validated to confirm that test results meet the requirements for evaluating safety in clinical use.[2,3] Negative and positive reference materials or positive control substances have been used in biological safety evaluation tests such as cytotoxicity, sensitization, genotoxicity, and implantation tests to detect toxicity in test samples and to certify the validity of the test system. However, no reference materials have been used for hemolysis testing to date.

Hemolysis is a phenomenon accompanied by the rupture of erythrocytes and the release of their cytoplasm into the surrounding fluid; thus, the cell membrane is damaged by physical, chemical, and biological factors. Certain types of snake and vespid venoms[8,9] with phospholipase A_2 activity and bacterial exotoxins[10–12] produced by pathogenic microbes (e.g., *Clostridium perfringens*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*) are known as typical hemolysins, but considering the safety, stability, and cost performance of potential positive reference materials, a candidate hemolytic substance should be selected from commercially available chemical compounds.

In this study, we developed a positive reference material made of a plasticized polyvinyl chloride (PVC) sheet that shows strong or weak hemolytic activity dependent on the content of Genapol X-080, selected as a candidate hemolytically toxic substance, based on results obtained using 23 types of chemical compounds, and we evaluated the performance of this material as a positive reference material in three types of official test methods used in Japan and the USA.

MATERIALS AND METHODS

Materials, chemicals, and utensils

Twenty-three types of chemicals selected as candidate hemolytic compounds and their suppliers are listed in Table I. Dimethyl sulfoxide (DMSO) of molecular biology grade, sodium citrate, sodium carbonate, ammonium thiocyanate, cobalt (II) nitrate hexahydrate, nitric acid for analysis of poisonous metals, metal standard solution (Co and Ag), and Dulbecco's phosphate-buffered saline (PBS) without Ca^{++} and Mg^{++} were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Toluene of pesticide residue and PCB analysis grade and Drabkin reagent were purchased from Sigma-Aldrich (Tokyo, Japan), and 4-(2-pyridylazo)-

resorcinol (PAR) was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). The hemoglobin standard was purchased from Alfresa Pharma Corporation (Osaka, Japan). All utensils made of glass, metal, or Teflon® used to prepare samples for inductively coupled plasma mass spectrometry (ICP-MS) analysis were heated at 250°C for more than 16 h prior to use.

Animal blood

The rabbit defibrinated blood used in simple hemolysis testing for a candidate hemolytic compound as described below was purchased from Kohjin Bio (Saitama, Japan). For hemolysis tests provided by the Japanese Ministry of Health, Labor, and Welfare (MHLW), rabbit defibrinated blood was prepared in our laboratory according to Japanese guidelines.[7] For hemolysis tests provided by the American Society of Testing and Materials (ASTM) and the National Institutes of Health (NIH),[5,6] fresh-drawn rabbit blood was anticoagulated by adding sodium citrate at a final concentration of 0.38 w/v% and stocked at 4°C until use. New Zealand white rabbits and Japanese white rabbits were used in this study, and the drawn blood was directly used for hemolysis testing without washing the blood cells. The procedure was performed in accordance with the ethical guidelines on animal experiments of the Hatano Research Institute of the Food and Drug Safety Center (approval number 1120331A) and Terumo Corporation (approval number 120163).

Preparation of PVC sheet

PVC powder (100 g) was added gradually to a mixture of DEHP (55 g) and ESBO (8 g) containing different amounts of Genapol X-080 or diphenyl disulfide (0, 0.01, 0.1, 1.0, and 10 g, respectively; final concentration = 0, 0.006, 0.06, 0.6, and 5.8% [w/w]) under stirring with a spatula. The mixed powder was gently heated from room temperature to 100°C in an oven and then stirred well. The powder was stirred a second time after heating at 100°C for 5 min to completely plasticize the PVC. The plasticized powder was heat-pressed at 180°C to prepare the PVC sheets (thickness = 0.4 mm). Each sheet was cut into small pieces (1 × 3 cm^2).

Simple hemolysis testing to survey a candidate hemolytic compound

Purchased rabbit defibrinated blood (1 mL) was placed into a screw-capped test tube and centrifuged at 700*g* for 5 min at 4°C. The supernatant was removed, and PBS (10 mL) was added to the sedimented red blood cells (RBC) and then gently mixed. After centrifugation at 700*g* for 5 min at 4°C, the supernatant was removed, and this step was repeated two times to wash RBCs. The RBC sediment was finally suspended in PBS of equal volume to original sample and stored at 4°C until use.

Hydrophilic compounds were dissolved in PBS (1 mL) at the final concentration shown in Table I, and the washed RBC suspension (20 μL) was added to the solution and then gently mixed. After incubation at 37°C for 1, 2, and 4 h, each sample was centrifuged at 700*g* for 5 min at 4°C, and then the absorbance of the supernatant (100 μL) was measured at 415 nm using a SH-9000 Lab microplate reader (Corona Electric, Ibaraki, Japan). The RBC suspension in PBS alone was also tested under the same conditions as a negative control, whereas a positive control was prepared using distilled water instead of PBS. The calculated amount of the hydrophobic compound was dissolved in DMSO, and the solution was diluted 100-fold with PBS at the final concentration listed in Table I. The washed RBC suspension (20 μL) was immediately added to the diluted sample (1 mL), and then hemolytic behavior was estimated using the same method.

The hemolytic behavior of Genapol X-080 and diphenyl disulfide exhibiting high hemolytic activity against RBC was additionally estimated in detail at concentrations from 0.1 to 50 μg/mL for Genapol X-080 and from 50 to 100 μ*M* for diphenyl disulfide.

These tests were performed in duplicate. The hemolytic ratio was calculated in accordance with the following formula.

$$\%\text{Hemolysis} = \frac{(A_T - A_N)}{(A_P - A_N)} \times 100.$$

where A_T is the test sample absorbance, A_N is the average negative control mean absorbance, and A_P is the average positive control mean absorbance.

Hemolysis testing of PVC sheet spiked with hemolytic compound

This test was performed using four methods: a simple method used to survey a candidate hemolytic compound, and official methods provided by ASTM, NIH, and Japanese MHLW guidelines.[5–7] PVC sheets (5 g for the NIH method or 1 × 3 cm^2 for the other methods) containing different amounts of Genapol X-080 or diphenyl disulfide were cut into small pieces and placed into a screw-capped glass bottle. A PVC sheet containing no Genapol X-080 or diphenyl disulfide was used as a negative material control.

In the simple method, 1 mL of PBS and 20 μL of washed, suspended RBCs prepared according to the procedure described above were added to the bottle containing the test pieces for the direct contact hemolysis test. In the extraction method, 1 mL of PBS was added to the bottle and autoclaved at 121°C for 1 h to make the test solution, and then the washed RBC suspension (10 μL) was added to 0.5 mL of each test solution replaced into another glass tube. After incubation at 37°C for 1, 2, and 4 h, the hemolytic ratio of each sample was measured according to the same method described above.

For ASTM direct contact hemolysis testing, PBS (7 mL) and 1 mL of rabbit blood anticoagulated with sodium citrate were used to adjust the hemoglobin concentration to 9.456 mg/mL, and they were added to the bottle containing sample pieces. For the ASTM extract method, PBS (8 mL) was added to the bottle and autoclaved (121°C, 1 h) to prepare the test solution. Citrated rabbit blood (1 mL) was added to 7 mL of the test solution replaced into another glass bottle. Each sample was incubated at 37°C for 3 h under gentle

mixing followed by centrifugation at 800*g* for 15 min. The supernatant (1 mL) was mixed with Drabkin reagent (1 mL) and the absorbance was measured at 540 nm after incubation at room temperature for 15 min. The hemoglobin concentration in each sample was calculated according to a standard curve.

For the NIH direct contact method, rabbit blood (8 mL) anticoagulated with sodium citrate was diluted with saline (10 mL). The diluted blood (0.2 mL) was hemolyzed by adding 0.1 w/v% sodium carbonate solution (10 mL), and the absorbance at 545 nm was adjusted to the range of 0.9–1.0 by adding saline or blood. Saline (10 mL) was added to the bottle containing the sample pieces and pre-incubated at 37°C for 30 min. The blood adjusted according to the absorbance (0.2 mL) was added to the glass tube and incubated at 37°C for 1 h. After centrifugation at 500*g* for 5 min, the absorbance of the supernatant was measured at 545 nm.

For the Japanese MHLW extract hemolysis test, the test solution was prepared by autoclave extraction (121°C, 1 h) with saline, and 0.2 mL of rabbit defibrinated blood freshly prepared in our laboratory was added to 10 mL of the test solution, followed by incubation at 37°C for 4 h. After centrifugation at 750*g* for 5 min, the supernatant (0.5 mL) was mixed with Drabkin reagent (4.5 mL) and incubated at room temperature for 20 min followed by measurement of the absorbance at 540 nm.

Negative and positive controls and the hemolysis ratio were prepared or calculated in accordance with the method provided by the guidelines. These tests were performed in triplicate, and significant differences were calculated by two-way analysis of variance.

Elution testing of Genapol X-080 from the PVC sheet

The amount of Genapol X-080 eluted from the PVC sheet was quantified by ICP-MS analysis as the amount of cobalt (II)-formed complex produced according to a previously reported method.[13,14] Briefly, 1 mL of PBS and 20 μL of washed RBC suspension were added to the bottle containing the test pieces of the PVC sheet (1 × 3 cm^2) spiked with different amounts of Genapol X-080. After incubation for 1, 2, and 4 h, 500 μL of each sample solution was replaced into another screw-capped glass tube, and 500 μL of test solution prepared by autoclave extraction of the test pieces with PBS (1 mL) was also sampled into another tube. PBS (0.5 mL), toluene (6 mL), and a mixture (3 mL) of ammonium thiocyanate (456 g/L) and cobalt (II) nitrate hexahydrate (46.6 g/L) solutions (1:1, v/v) were added to each sample and shaken for 15 min at room temperature followed by centrifugation at 2500 rpm for 3 min. PAR solution (1 mL, 0.01 g/L, pH 9.5) was added to 5 mL of the supernatant (toluene layer), and gently shaken for 3 min followed by centrifugation. The water layer was sampled for ICP-MS analysis. PBS containing Genapol X-080 in a concentration range from 1.0 to 200 μg/mL was also treated using the same method to generate a standard curve. This test was repeated in triplicate.

ICP-MS analysis

The sample solution was diluted appropriately 100–400-fold with 5 v/v% nitric acid and filtered through a membrane filter (pore size 0.45 μm, Sartorius Stedim, Goettingen, Germany). The concentration of Co ($m/z = 59$) in the sample solution was determined by ICP-MS (Agilent 7500ce, Agilent Technologies, Santa Clara, CA) using an octopole reaction system.[15,16] Helium gas was used as the collision gas. The operation conditions of ICP-MS were as follows: RF power 1500 W; argon plasma gas 15 L/min; nebulizer gas 0.7 L/min; make-up gas 0.33 L/min; helium collision gas 5 mL/min; spray chamber temperature 2°C; integration time 0.1 s; three replicates. Ag ($m/z = 107$) was used as an internal standard (5 μg/L in 5% nitric acid solution) for quantification of the Co concentration. The method limit of detection (LOD) and limit of quantification (LOQ) for Co in the final sample solution were calculated as follows[17]:

$$\mathrm{LOD} = 3 \times \rho$$

$$\mathrm{LOQ} = 14.1 \times \rho$$

where ρ is the standard deviation obtained from the results of a control blank sample (only PBS, $n = 10$). The LOD and LOQ of Co thus obtained were 6.67 and 31.7 ng/L, respectively. The background equivalent concentration (BEC) of Co in this study was 1.25 ng/L. Linearity ($r = 0.997$) of the standard curve used to quantify Genapol X-080 was observed in the concentration range from 1.0 to 200 μg/mL, and hence, the LOQ for Genapol X-080 was set at 1.0 μg/mL.

RESULTS

Hemolytic activity of a candidate compound

The 23 chemicals listed in Table I were selected as candidate hemolytic compounds according to previous reports,[18–20] and the hemolytic behavior against rabbit RBCs was estimated using a simple survey method. As shown in Table I, although most of the compounds did not exhibit hemolytic activity under the conditions used in this study, Genapol X-080 and diphenyl disulfide significantly induced hemolysis. As shown in Figure 1(a), the activity of Genapol X-080 remarkably increased at concentration higher than approximately 20–25 μg/mL. Diphenyl disulfide exhibited hemolytic activity in a concentration-dependent manner in the concentration range from 50 to 100 μ*M* [Figure 1(b)].

Preliminary study on the hemolytic activity of the PVC sheet spiked with Genapol X-080 or diphenyl disulfide

To confirm its availability as a positive reference material, the hemolytic behavior of PVC sheets spiked with different amounts of Genapol X-080 or diphenyl disulfide was preliminarily estimated using a simple method.

As shown in Figure 2(a), although PVC sheets containing 0.006% or 0.06% (w/w) Genapol X-080 did not exhibit significant hemolytic activity when compared with PBS (control) and the negative material control, the PVC sheet spiked with 0.6% (w/w) Genapol X-080 induced weak hemolysis of RBCs in the direct contact method, and the hemolytic ratio reached 37.4% after 4 h of incubation. In addition, complete hemolysis was induced by the PVC sheet containing 5.8%

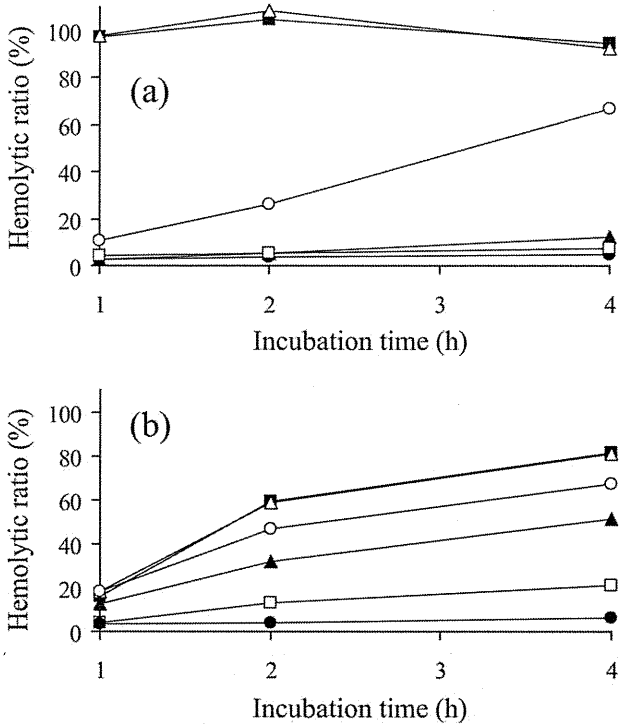

FIGURE 1. Hemolytic behavior of Genapol X-080 (a) and diphenyl disulfide (b) estimated by the simple method. Concentration of Genapol X-080: ●, 1; □, 10; ▲, 20; ○, 25; △, 30; ■, 40 μg/mL. Concentration of diphenyl disulfide: ●, 50; □, 60; ▲, 70; ○, 80; △, 90; ■, 100 μ*M*. The hemolytic ratio of negative control (PBS) after incubation for 1, 2, and 4 h was 4.2%, 4.8%, and 7.2%, respectively.

(w/w) Genapol X-080 independently of incubation time. Similar hemolytic behavior was observed for the extract-based method as shown in Figure 2(b), in which the test solution prepared by autoclave extraction of PVC sheets spiked with 0.6% or 5.8% (w/w) Genapol X-080 exhibited weak or strong hemolysis, respectively, but no activity was observed for extracts from PVC sheets containing 0.006% or 0.06% (w/w) Genapol X-080.

In contrast, the PVC sheet containing diphenyl disulfide did not exhibit hemolytic activity in the direct contact and/or extraction methods, despite the presence of the spiked diphenyl disulfide as shown in Figure 3.

Performance evaluation of PVC sheet spiked with Genapol X-080 as a positive reference material

The hemolytic activity of PVC sheets spiked with Genapol X-080 was also estimated using three official methods to certify the sheet as a positive reference material for hemolysis testing. As shown in Figure 4, the hemolytic behavior of the sheet detected by the ASTM direct contact method was almost identical to that in the simple method used in the preliminary survey, in which the PVC sheet containing 0.6% or 5.8% (w/w) Genapol X-080 induced weak (hemolytic ratio = 21.1%) or strong (hemolytic ratio = 92.4%) hemolysis, respectively, but no hemolysis for observed for the other two sheets spiked with relatively low amounts of the compound. Similar hemolytic behavior was also yielded by the ASTM or Japanese MHLW extract-based method and the NIH direct contact method, in which PVC sheets spiked with the highest amount of Genapol X-080 induced complete hemolysis (Figure 4). However, a PVC sheet spiked with 0.6% (w/w) Genapol X-080 did not exhibit significant hemolytic activity, which was different from the results in the ASTM direct contact method and the simple method used to survey the candidate hemolytic compound.

Amount of Genapol X-080 eluted from the PVC sheet

The amount of Genapol X-080 eluted from the PVC sheet was measured by ICP-MS analysis to elucidate the correlation with the hemolytic behavior of the sheet. In the direct contact method, the amount eluted from PVC sheets spiked with 0.6% or 5.8% (w/w) Genapol X-080 increased over time and reached 25.6 or 1154 μg/mL, respectively, after 4 h of incubation as shown in Table II. The test solution prepared by autoclave treatment for the extract-based method contained 22.5 (former sheet) or 358 (later sheet) μg/mL Genapol X-080, respectively. In contrast, the amount eluted from the PVC sheet containing the lowest amount of Genapol X-080 was less than the LOQ in the direct contact and/or extract-based methods. No significant elution was observed from the PVC sheet spiked with 0.06% (w/w) Genapol X-080, except for 3.75 μg/mL Genapol X-080 detected after incubation for 4 h using the direct contact method.

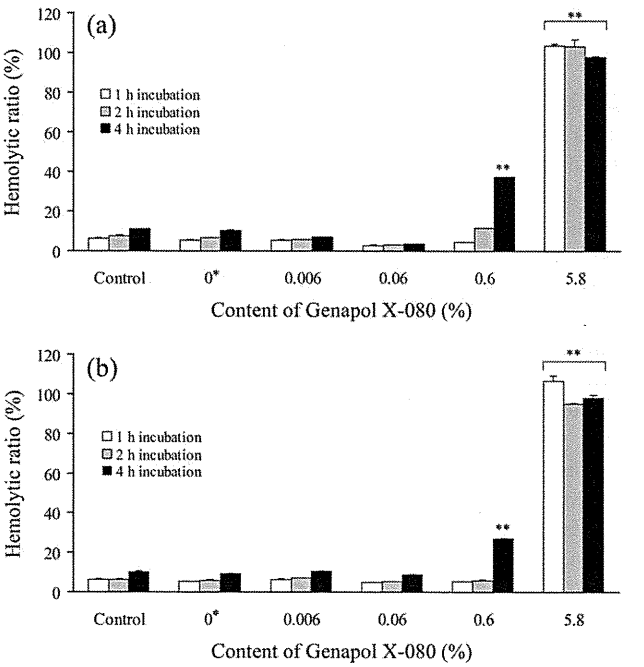

FIGURE 2. Hemolytic behavior of PVC sheets spiked with different amounts of Genapol X-080 estimated by direct contact (a) and extract-based (b) assays using the simple method. In addition to PBS alone (negative control), a *PVC sheet containing no Genapol X-080 was used as a negative material control. **Significant differences ($p < 0.01$) were detected between these data points and other data points.

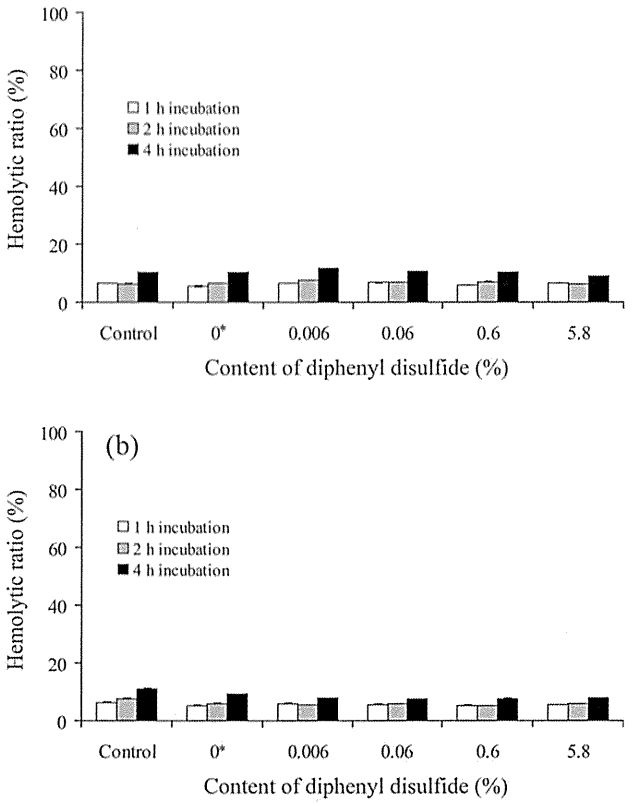

FIGURE 3. Hemolytic behavior of PVC sheets spiked with different amounts of diphenyl disulfide estimated by direct contact (a) and extract-based (b) assays using a simple method. In addition to PBS alone (negative control), a *PVC sheet containing no diphenyl disulfide was used as a negative material control.

DISCUSSION

This investigation attempted to develop a positive reference material for certifying the validity of hemolysis tests, which are used for evaluating the hemocompatibility of medical devices. From the survey of 23 chemical compounds, Genapol X-080 and diphenyl disulfide were selected as candidates.

Genapol X-080 is a polyethyleneglycol (PEG) monoalkyl ether that is used as a general-purpose non-ionic detergent, and it has been widely used in various fields including cosmetics. Alkyl PEG ethers were not genotoxic at a concentration of 1000 mg/L,[21] and no evidence of systemic toxicity or sensitization was found for intact skin.[22] Recently, the Cosmetic Ingredient Review (CIR) Expert Panel assessed the safety of alkyl PEG ethers used in cosmetics and concluded that they are safe as used in cosmetic products.[23] Taking these findings into consideration, it seems that this compound is safe for handling and useful as an ingredient for preparing a positive reference material for hemolysis testing. Genapol X-080 induces hemolysis of RBCs by destroying the cell membrane through the detergent effect at concentrations higher than the critical micelle concentration (CMC), which may be approximately 25 μg/mL according to the hemolytic behavior of the compound [Figure 1(a)]. PVC sheets spiked with 0.6% or 5.8% (w/w) Genapol X-080 exhibited weak or strong hemolysis, respectively, in the direct contact and/or extract-based assays using the simple method. In the direct contact method, the amount of Genapol X-080 eluted from the PVC sheets increased over time and reached 25.6 (0.6% [w/w] sheet) and 1154 (5.8% [w/w] sheet) μg/mL after 4 h incubation, corresponding approximately to the CMC or much higher than the CMC, respectively. The Genapol X-080 content in the test solution prepared by autoclave extraction was also similar to or higher than the CMC. Thus, a clear relationship was observed between the degree of hemolytic activity and elution amount of Genapol X-080. In addition, identical or similar hemolytic behavior was also yielded by the ASTM, NIH, and Japanese MHLW methods according to the protocol for inter-laboratory hemolysis round robin testing produced by ISO/TC 194/WG 9, which was implemented in April 2013. Although weak hemolytic activity induced by the PVC sheet spiked with 0.6% (w/w) Genapol X-080 was not detected by official methods other than the ASTM direct contact method, the detection may be improved by slightly increasing the spiked amount of the compound. The Genapol X-080 content in the test solution prepared by autoclave extraction of the PVC sheet spiked with the highest amount of the compound was considerably lower than the amount eluted from the sheet during the hemolysis test using the direct contact method. The difference in elution amount may originate from the physicochemical properties of Genapol X-080 because the solubility of the compound markedly decreases at temperatures higher than the cloud point (74°C–76°C). These data clearly indicate that a PVC sheet spiked with Genapol X-080 exhibiting different hemolytic activity dependent on the Genapol X-080 content may be useful as a positive reference material to certify the validity of hemolysis tests.

It has been reported that aromatic disulfides induce hemolysis of erythrocytes,[19,20] and in fact, diphenyl disulfide itself also exhibited significant hemolytic activity in this study. This compound induces hemolysis as the result of biological effects in erythrocytes such as hydrogen

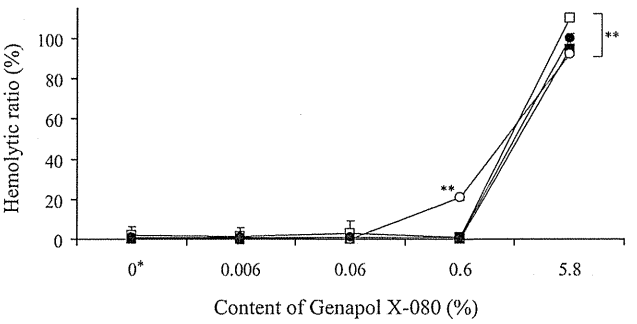

FIGURE 4. Hemolytic behavior of PVC sheets spiked with different amounts of Genapol X-080 estimated by official methods for hemolysis testing provided by ASTM, NIH, and the Japanese MHLW. ■, ASTM extract-based method; ○, ASTM direct contact method; □, NIH direct contact method; ●, Japanese MHLW extract-based method. *PVC sheet containing no Genapol X-080 was used as a negative material control. **Significant differences ($p < 0.01$) were detected between these data points and other data points.

TABLE II. Amount of Genapol X-080 Eluted from PVC Sheet During Hemolysis Test with Direct Contact Method or into Test Solution for the Extract Method

Genapol X-080 content in PVC sheet (% [w/w])	Amount of Genapol X-080 (μg/mL) eluted from PVC sheet in			
	Direct contact method after incubation for			
	1 h	2 h	4 h	Extract method
0.006	-[a]	-	-	-
0.06	-	-	3.75±0.12	-
0.6	6.20±0.21	21.2±0.43	25.6±0.70	22.5±0.32
5.8	362±5.0	523±14.8	1154±31.6	358±6.4

[a] less than LOQ.

peroxide production, hemoglobin oxidation, and destruction of nonprotein thiols and protein-bound thiols.[24] Although diphenyl disulfide itself seemed to be superior to Genapol X-080 as a candidate hemolytic compound with regard to the wide range of concentrations that induced hemolysis, PVC sheets spiked with diphenyl disulfide exhibited no hemolytic activity in the direct contact and/or extract-based methods. The reason is unknown, but it is speculated that although diphenyl disulfide itself may be heat-stable, because its hemolytic activity was maintained even after heating at 200°C for 10 min (data not shown), it may interact with DEHP, ESBO, or PVC molecules during the heat-press process used to make the PVC sheet. In fact, PVC sheets prepared by casting a tetrahydrofuran solution containing the calculated amounts of plasticized PVC and diphenyl disulfide induced hemolysis of RBCs (data not shown). However, it was finally decided that the PVC cast sheet was not useful as a positive reference material for hemolysis testing because the degree of hemolytic activity was independent of the spiked amount and the reproducibility of the data was low (data not shown), probably because the amount of diphenyl disulfide eluted from the PVC sheet may have varied according to the hydrophobicity of the compound.

It is known that most plastic products do not exhibit significant hemolytic activity against RBCs. In fact, we have also confirmed that no hemolytic activity is present in commercially available plastic sheets made from polycarbonate, acrylate, and terephthalate (data not shown), which indicates that these sheets can be used as a negative reference material for hemolysis testing. Although there is a possibility that the method used to form the sheets will be changed suddenly without any publicity, plastic products that can be used as a negative reference material may be easily found and purchased at any time. Thus, the use of efficient positive and negative reference materials to certify the validity of hemolysis tests is now possible, and our next step is to establish a reliable supply of a positive reference material containing Genapol X-080.

CONCLUSIONS

PVC sheets spiked with Genapol X-080 exhibiting different hemolytic activity dependent on the Genapol X-080 content may be useful as a positive reference material to certify the validity of hemolysis tests.

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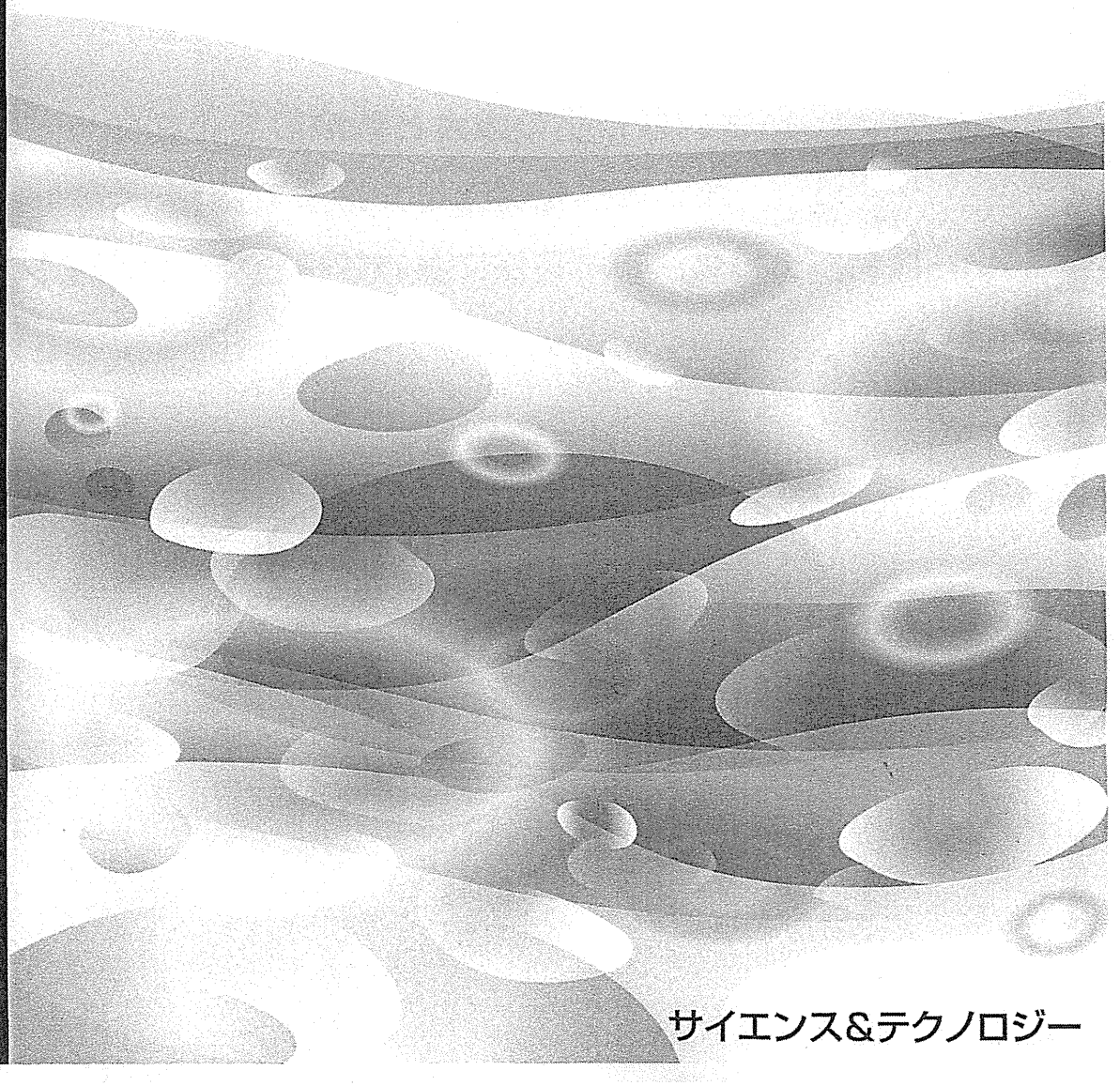
Ⅲ．研究成果の刊行に関する資料⑥

生体適合性制御と要求特性掌握から実践する高分子バイオマテリアル
の設計・開発戦略

生体適合性制御と要求特性掌握から実践する
高分子バイオマテリアルの設計・開発戦略

モノマー（いち）からデザインするバイオインターフェースと上市までの道筋



サイエンス&テクノロジー

第 1 部

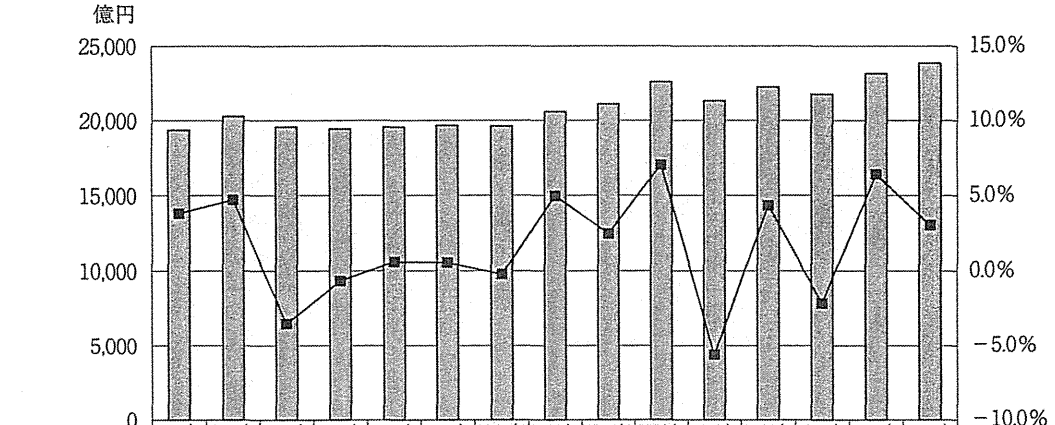
医療機器市場の拡大と新規製品の開発
及び実用化促進を支援する施策
―開発，上市化，市場確保において留意すべきポイント―

国立医薬品食品衛生研究所　　葩島 由二

はじめに

我が国の医療機器市場規模は，平成12年度から平成15年度まで横這いで推移していたが，平成16年以降増加し，平成23年は約2.4兆円となり過去最大となった(図1)。平成23年の医療機器市場において，金額ベースでは治療機器(カテーテル，ペースメーカ等)が53%，診断機器(内視鏡，CT，MRI等)が26%を占めており，一般的に治療機器の成長率が高く，市場規模も大きい(図2)。医療機器市場は景気の影響を受け難く，安定した需要がある。平成22年度における我が国の医療費は37.4兆円であり，医療機器市場はその6%強を占めている[1)]。GDPに占める医療費の割合は，現在8%から15%であるが，2050年には20%から36%(米国：36.1%，ドイツ：25.9%，日本：21.9%)と大幅な増加が予測されている。経済協力開発機構(OECD)でのデータにおいても，医療費の総額は1.9%の増加を示しており，米国を除けば，2010年ではGDP比約9%となっている。

高齢化の進展と新興国における医療需要拡大を受け，医療機器の国際市場は急成長しており，世界的競争が激化しているが，日本市場においては年々輸入が拡大しており，2010年では0.6兆円の貿易赤字となっている。貿易黒字額の多い医療機器としては，血液検査装置，採血管・輸血バッグ，透析器，医用写真フィルム，全身用X線CT装置等があり，逆に貿易赤字



	H9年	H10年	H11年	H12年	H13年	H14年	H15年	H16年	H17年	H18年	H19年	H20年	H21年	H22年	H23年
国内市場[億円]	19,373	20,286	19,573	19,443	19,558	19,666	19,622	20,596	21,105	22,587	21,314	22,239	21,760	23,154	23,860
対前年伸び率	3.8%	4.7%	−3.5%	−0.7%	0.6%	0.6%	−0.2%	5.0%	2.5%	7.0%	−5.6%	4.3%	−2.2%	6.4%	3.0%

出典：厚生労働省　薬事工業生産動態統計

図1　我が国の医療機器の市場規模と対前年伸び率の推移[23)]

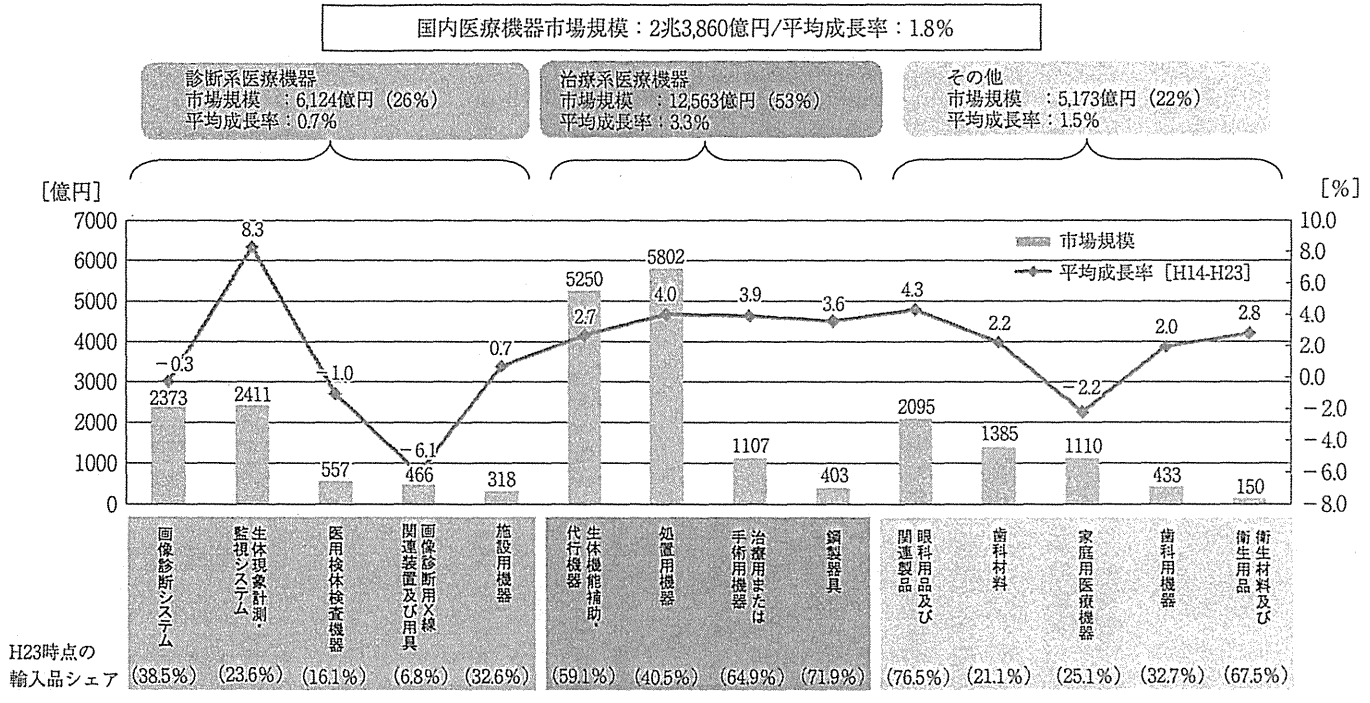


図2　日本の医療機器市場の構造[23]

注：国内市場規模＝国内生産額＋輸入額－輸出額　　「平成23年薬事工業生産動態統計年報」をもとに作成

となっている代表的な製品として，コンタクトレンズ，人工関節・人工骨，ステント，血管用チューブ・カテーテル，縫合用器械器具等が挙げられる(表1)。

我が国のものづくり企業は医療機器に活かすことができる高い技術力を持っているが，このような貿易収支を見る限り，その力が十分に活用されていないのが現状である。これを改善するためには，医療イノベーションの推進に関する取り組みが不可欠である。世界的に医療機器開発の環境は厳しい局面を迎えており，グローバルな企業間競争に勝ち残るためには，よりイノベーティブな医療機器が患者に迅速に提供されることが重要である。医療イノベーションへの期待は国内にとどまらず，世界規模のニーズでもある。

ジェトロの調査結果(米国医療機器産業の活性化に向けた政府支援策と企業の事例：2012年3月)によると，米国医療機器産業(企業数は約7000社)では，主に中小企業(従業員20名以下の会社が全体の62%)がイノベーションを実現する役割を担い，大企業は中小企業から獲得した新技術を具体的な製品に仕上げて臨床試験を行い，承認を得て世界規模の販売網で市場展開している[2)]。活発な産学連携の中にあって，エンドユーザーである医師自身が医療機器開発に

表1　品目別の貿易収支の状況(それぞれ上位5品目)[23)]

貿易赤字額の多い医療機器

	品目	輸出額(億円)	輸入額(億円)	貿易赤字額(億円)
1	コンタクトレンズ	1	1,413	**1,412**
2	人工関節，人工骨及び関連用品	5	1,158	**1,153**
3	ステント	16	433	**417**
4	血管用チューブ及びカテーテル	200	565	**365**
5	縫合用器械器具等	0	349	**349**

貿易黒字額の多い医療機器

	品目	輸出額(億円)	輸入額(億円)	貿易黒字額(億円)
1	血液検査装置等	542	4	538
2	採血管，輸血バッグ等	488	44	444
3	透析器	304	26	278
4	医用写真フィルム	226	20	206
5	全身用X線CT装置	327	137	190

「平成23年薬事工業生産動態統計年報」より作成

深く関与しており，我が国においても医工連携の重要性が提唱されている。米国政府は研究開発助成や税制優遇，輸出支援，承認取得の迅速化等を行っているが，医療機器開発が盛んなマサチューセッツやミネソタ，カリフォルニア等の州政府も科学研究支援，企業支援，人材育成，設備投資支援等を行っている。米国は世界最大の市場を抱え，多額の予算と人的資源，競争力を生み出す開発環境で世界をリードしている。

欧州5か国の2005年から2009年までのCAGR(年平均成長率)の伸びは，ドイツ2%，フランス4%，英国7%，イタリア3%，スペイン10%であったが，2010-2011年では大きく落ち込んでいる。欧州における2004年から2011年の医療機器の価格を見ても，平均としてMRI/5%，CT/5%，IVポンプ/8%，モニタ/5%，ペースメーカ/8%，ステント/34%と下落している。また，1995年から2010年にかけての研究開発投資は8%から7%へとダウンしている。このようなことから，欧州では医療技術の真の価値を創成するための施策として，医療の変化に対するイノベーション，コストベースのイノベーション，価値を生み出すイノベーション等を真剣に考えている。

このように世界的な医療技術イノベーションの競争の中，我が国におけるイノベーションの成果を世界へ発信すると共に，医療産業を日本経済の新たなけん引役に成長させることを目指して，国際市場を占有できる高品質・高機能製品の開発と実用化促進を支援するさまざまな政策が国家戦略として推進されている。

1. 日本発の新規医療機器・医用材料の開発を支援する国家戦略

規制システムは開発及び市場アクセスの重要な要素であり，投資判断にも影響を与えている。2011年のPWCによるMedical Technology Innovation Scorecardによると，イノベーションを推進するには五つの重要な柱(財政的なインセンティブ，イノベーション・リソース，規制フレームワーク，患者の要求，投資コミュニティ)があり，いかに規制がイノベーションに対してサポーティブかどうかが重要な因子の一つとなる。

医療機器開発に活用できる優れた技術が十分に活用されていない要因として，①現在の薬事法では必ずしも医療機器の特性を踏まえた規制体系になっていないため，開発に時間を要することがある，②生命や健康に直結する分野であり，参入リスクが高い，③医療現場が有する課題・ニーズがものづくり現場に行き届いていないこと等が挙げられる。このような問題は，医療現場の課題やニーズを選定後，優れたものづくり技術を有する中小企業等と医療機関等の間で医工連携体制を構築して医療機器の開発及び改良を促進すると共に，国内外の臨床評価から実用化までの一貫した取組をサポートすることにより解決されると思われる。医療イノベー

ション5か年戦略[3]の一環として，経済産業省，厚生労働省及び文部科学省が連携して実施している課題解決型医療機器等開発事業[4]や医療ニーズの高い医療機器等の早期導入に関する検討会[5]等における取組は，これを具現化した施策である。この他，新規医療機器の開発を支援するためにさまざまな施策が実施されている。次に主な施策の内容を概説する。

1.1 医療イノベーション5か年戦略

平成25年度に内閣官房医療イノベーション推進室が掲げた政策である医療イノベーション5か年戦略では，基礎研究から実用化に至るプロセスにおける技術の融合として，①医工連携による橋渡し支援の整備，②医工連携の医療機器開発支援，③臨床試験の拠点整備の他，医療現場のニーズに基づく改良・改善として，④医療機器の特性を踏まえた規制のあり方の検討，承認審査として，⑤迅速に審査できる体制整備，保険適用として，⑥イノベーションの適切な評価等が挙げられている。また，新たな産業として期待されている再生医療分野においても，①長期間を要する基礎研究への支援，②再生医療の特性を踏まえた規制のあり方の検討，③迅速審査できる体制整備，④インフラ等の国際標準化の取得，⑤再生医療関連産業の振興等の主な施策が挙げられており，開発期間の短縮，より早い患者への供給のためには，これらの施策を確実に実行していく必要がある。

1.2 薬事法改正[6]

医療機器の多くは短期間で改善・改良されると共に，そのリスク・ベネフィットバランスは使用者である医師の手技によるバイアスが入る点等について，医薬品と大きく異なる特性を持つ。平成25年度の改正を目指す薬事法では，医療機器の特性を踏まえた制度を創設するため，医療機器に関する条項を医薬品と切り離して別立てとする方向で協議されている。今回の改正では，単体プログラムの位置付け，製造販売業・製造業の許可の簡素・合理化，第三者認証機関における認証の対象拡大，QMS調査の合理化，再審査・再評価制度の見直し，運用の改善も掲げられている。また，同改正案では新たに再生医療製品の定義を置くことも検討されている。再生医療技術はリスクの程度に応じて，第一種(ES細胞，iPS細胞等)，第二種(体性幹細胞等)及び第三種(体細胞加工等)再生医療に分類される方向で議論されている。リスクの高い第一種再生医療の場合，医療機関が提供計画を作成し，特定認定再生利用等委員会の審査を受けた後，厚生労働大臣に提出して実施する。その後，一定の実施制限期間内に厚生労働大臣が厚生科学審議会の意見を聴取し，安全性等の基準に適合していないときは計画の変更を命令できる。第二種再生医療も特定認定再生利用等委員会の審査を受けるが，第一種と異なり，厚生科学審議会は関与しない。第三種については，特定認定再生利用等委員会に代わって認定再生

医療等委員会が審査を行う。再生医療規制法案は自由診療を含むヒト細胞を使用する全ての治療行為に倫理審査委員会の承認と国への届出を義務づけ，違反には治療中止命令を出すことができる内容となっている。薬事法改正案では，再生医療製品について，安全性が確認できれば，有効性が完全に証明されていない段階でも条件付きで国の承認が受けられる等，手続きを簡素化することも検討されている。

医療機器の開発や許認可等に要する時間は，平成17年度の薬事法改正や医療イノベーションの前身的施策である「革新的医薬品・医療機器創出のための5か年戦略(平成19年度)」等の成果の一つとして短縮されてきたが，今回の薬事法改正により，イノベーションを更に推進できる規制体系となることが期待される。

1.3 スーパー特区プロジェクト[7)]

内閣府科学技術政策・イノベーション担当部門は，革新的技術の開発を阻害している要因を克服するため，研究資金の特例や規制を担当する部局(医薬品医療機器総合機構：PMDA)との並行協議等を試行的に行う「革新的技術特区」，いわゆる「スーパー特区」を創設した。これは，従来の行政区域単位の特区でなく，テーマ重視の特区(複合体拠点の研究者をネットワークで結んだ複合体)である。平成20年度より，その第一弾として「先端医療開発特区」が創設され，最先端の再生医療，医薬品，医療機器の開発・実用化を促進している。

1.4 橋渡し研究加速ネットワークプログラム[8)]

アカデミア発の基礎研究成果を実際の医療に活用するための施策として文部科学省が平成19年度に創設した橋渡し研究支援推進プログラムは平成23年度をもって終了した。現在，この施策は第2期に移行し，橋渡し研究加速ネットワークプログラムとして継続されている。本施策は，画期的な医薬品・医療機器等を効率的・効果的に国民へ還元することを目指し，アカデミア発の有望な基礎研究成果の臨床研究・治験への橋渡しをさらに加速するため，全国7ヶ所に橋渡し研究支援拠点が指定され，シーズ育成能力を強化すると共に，恒久的な橋渡し研究支援拠点を確立することを目指している。

1.5 次世代医療機器評価指標作成事業/医療機器開発ガイドライン事業[9,10)]

数年後に実用化が期待される新しい医療機器(次世代医療機器)を医療現場へ迅速に導入することを目指した事業が経済産業省と厚生労働省の連携のもとに平成17年度から開始された。本事業は国内医療機器産業の活性化に資する施策の一環として実施されており，当該医療機器の開発段階から承認審査までを見通した施策として，経済産業省側(事務局：産業技術総合研