P1- 005

poly(Arg) - nanoparticle complexes translocate through lipidbilayer membranes.

Satoru Ueno, Saburo Shimabayashi

Sho-machi 1-78-1, Tokushima 770-8505, Japan

Introduction

Several reports have suggested that certain short natural and synthetic water-soluble cationic peptides which mainly translocate various cell membranes by endocytically show the non-endocytically translocation and we reported that cationic polypeptide: poly-L-arginine (poly(Arg)) and poly(Arg) - BSA complex directly translocate negatively charged liposome membrane. We have been investigating the translocationability of poly(Arg) - hydroxyapatite (hap) nanoparticle complex and poly(Arg) - Multi-walled Carbon Nanotube (mwct) complex, through negatively charged phospholipid (soybean phospholipid (SBPL)) bilayer membranes by several instruments.

Materials and methods

The Poly-L-arginine (poly(Arg): mw 35500), Soybean phospholipids (SBPL), and hap nanoparticles were purchased from Sigma (St. Louis, MO, USA). The mwct was purchased from TCI (Tokyo, Japan). The mwcts were dispersed by modified Hu et al. methods[1]. We have studied of the complexation of poly(Arg) and nanoparticles by zeta-potential measurement and the transfer of complexes across the liposome membrane by fluorescence, CD, and conforcal laser microscope (CLSM).

Results and Discussion

After the hap or mwct dispersion was mixed with poly(Arg) solution, the zeta-potentials were positively sifted. This zeta-potential shift was concluded adsorption of poly(Arg) on hap or mwct surface and the formation of poly(Arg) - hap or poly(Arg) - mwct complex. By the CLSM measurement, We find that both complexes can translocate through the negatively charged liposome membranes (Fig. 1).

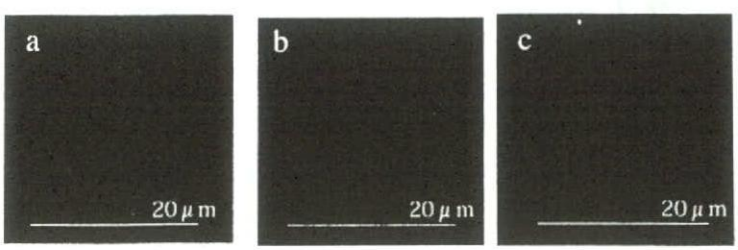

Figure 1. CLSM images of the distribution of poly(Arg) - hap complex: The complex and liposome were mixed after 1 min (a), mixed after 10 min (b) and mixed after 15 min (c).

Recently, we reported that the poly(Arg) - BSA complex was collapsed after membrane translocation, but the double stained CLSM measurement for poly(Arg) - hap complex shows that the complexes were retained in the inner water layer of SBPL liposome.

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Accelerated blood clearance (ABC) phenomenon on PEGylated nanocarriers: Unexpected immune-reaction against PEGylated liposomes

Taro Shimizu, Kosuke Nawata , Tatsuhiro Ishida, Hiroshi Kiwada

Department of Pharmacokinetics and Biopharmaceutics, The University of Tokushima, 1-78-1, Sho-machi, Tokushima 770-8505, Japan.

Abstract

Polyethylene glycol (PEG) is considered as non-toxic and non-immunogenic material, and surface modification with it can improve the immunogenicity and pharmacokinetics of nanocarriers. However, we recently reported that PEG-modified liposome (SL), which has been approved for clinical use, loses their long circulating properties when they are administered twice in same animal with certain interval (accelerated blood clearance (ABC) phenomenon) (Fig. 1) [1]. We elucidated that anti-PEG IgM, secreted in response to the first dose of SL, is responsible for the rapid clearance of the second dose via initiation of complement activation [2]. We further elucidated that such accelerated clearance of SL is caused in nude mice (no T-cells) [3], while it was not caused in splenectomized rat (no spleen) [4]. These suggest that spleen produces the anti-PEG IgM in a T-cell independent manner. It appears that SL activates the immunity in spleen as T-cell independent antigens do. Our studies clearly demonstrate that any PEGylated formulations may display unexpected pharmacokinetic behavior upon repeated injection and, as a consequence, may show less therapeutic efficacy or even cause undesirable side-effects. Therefore, a strategy to abrogate the immunogenicity of PEGylated formulation without significant compromising their in vivo performance would be highly desirable for the further development of promising PEGylated formulations.

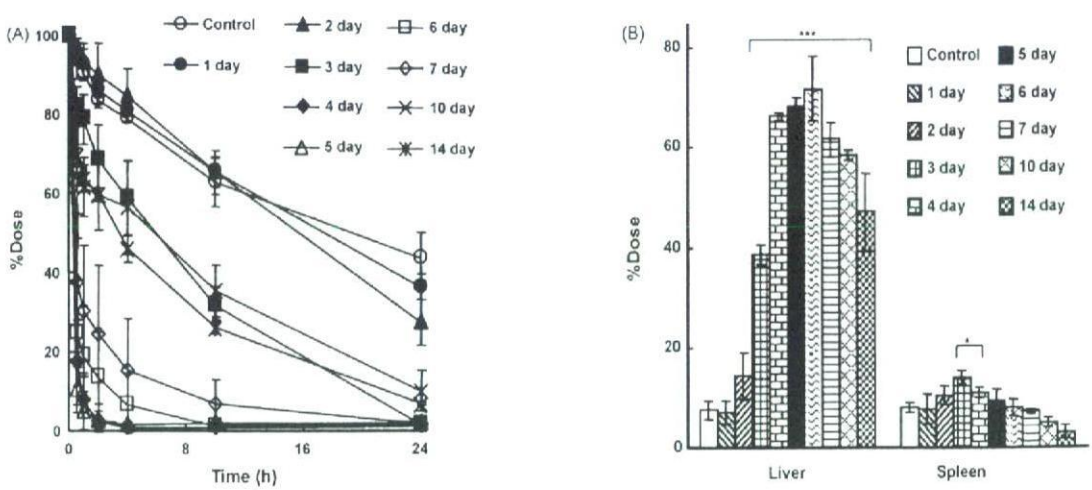

Fig. 1 Accelerated blood clearance and enhanced organ uptake of second dose of SL

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P1- 007

Effect of Lubricant on Wear Debris of Ultra High Molecular Weight Polyethylene Cups against Zirconia's Balls in Hip Joint Simulator

Masami Hashimoto[1], Mineo Mizuno and Satoshi Kitaoka

Japan Fine Ceramics Center, Nagoya, Japan, 2-4-1 Mutsuno, Atsuta-ku, Nagoya, 456-8587, Japan
Tel. +81-52-871-3500, Fax +81-52-871-3599, [1]masami@jfcc.or.jp

Introduction

The ultimate goal in the design of a hip joint wear simulator and an associated test protocol is to produce the type and amount of wear that occurs clinically. Hip simulators that are commonly used vary markedly in load and motion characteristics, and use various lubricants, including distilled water, physiological saline, blood serum and synthetic serum. At present, international standard recommends calf or bovine serum at pure concentration or diluted with distilled water as the best substitute for synovial fluid in *in vitro* tests, but there is no complete agreement concerning this choice. Owing to the importance of particle characteristics in the quantity and quality of biological responses, great emphasis has been given on developing techniques for extracting and studying the quantity, size and morphology of UHMWPE wear particles.

In this study, we analyzed UHMWPE particles using computerized image analysis to generate quantitative dimensions, and then categorize particles using subjective responses from individuals. Quantitative analysis was then performed to develop a computational model, that is, useful in classifying particles according in appropriate categories. This information may be useful for developing a common vocabulary for describing particles that incorporates both formal dimensions and subjective impression.

Materials and Methods

To assess the lubricity and production of wear debris, we performed hip simulator tests (5×10^6 cycles) using ultra high molecular weight polyethylene (UHMWPE) cup against 26 milimeter ZrO_2 heads. The base lubricants used were calf-sera from two companies: Sigma-Aldrich Japan (Serum A) and BioWest (Serum B) (Table 1). To examine wear debris, we extracted UHMWPE debris from the serum lubricant used in the simulator testing. UHMWPE debris analysis was conducted on a computer using a custom application based on the public domain image-processing and analysis program, Image J.

Table 1 Compositions of 25 vol% concentrations of serums used to lubricate wear tests

	Protein (Total)	Albumin	Globulin			Alb/G	Cholesterol
			α-G	β-G	γ-G		
Serum A	18.3	8.4	2.7	3.4	3.8	0.85	0.91
Serum B (mg/ml)	15.5	7.8	2.3	4.0	1.4	1	1.99

Results and Conclusions

Table 2 shows the change in the wear rate of the UHMWPE cups in the two serums. We defined the initial wear rate as that from the first cycle up to 1.0×10^{6th} cycle and steady wear rate as that from the 4.0×10^{6th} to the 5.0×10^{6th} cycle. The initial wear rate of the UHMWPE cup in serum A was 27.9 mg/10^6 cycles; the steady wear rate of the same UHMWPE cup increased to 41.41 mg/10^6 cycles. In contrast, those of the UHMWPE cup in serum B decreased to 16.93 and 12.82 mg/10^6 cycles, respectively.

Table 2 Wear rates of UHMWPE cup in hip joint simulator tests

Serum	Initial wear rate (mg/10^6 cycles)	Steady wear rate (mg/10^6 cycles)
Serum A	27.9	41.41
Serum B	16.93	12.82

SEM photographs of UHMWPE debris revealed that the number and area of small submicron –sized granules isolated from serum A was larger than that of wear debris isolated from serum B. This result is consistent with the wear rate result. And also, elongated wear particles were existed in serum A.

This hip joint simulator study demonstrated that the not only protein concentration but also cholesterol of a serum has marked effect on the wear performance of UHMWPE cups. As albumin/globulin protein ratio increased, UHMWPE wear rate decreased. A comparison of the quantitative measurements of the isolated UHMWPE debris from two serums showed that the amount of wear considerably differed between the serums. However, the types of UHMWPE debris that formed in the two serums were almost the same.

Genotoxicity of nanoparticles in in vivo mutation assay systems

Yukari Totsuka[1], Takamichi Ichinose[2], Kyoko Hiyoshi[3], Tatsuya Kato[4], Shuichi Masuda[4], Takehiko Nohmi[5], Takashi Sugimura[1], Keiji Wakabayashi[1]

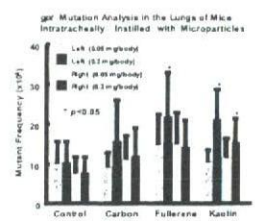
[1]Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 1-1, Tsukiji 5-Chome, Chuo-ku, Tokyo, 104-0045, Japan.

[2]Department of Health Sciences, Oita University of Nursing and Health Sciences, 2944-9 Megusuno Oita City, Oita, 870-1201, Japan.

[3]Fundamental Nursing, School of Nursing, [4]Department of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1, Yada, Shizuoka, 422-8526, Japan.

[5]Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan.

Recently, manufactured nanoparticles such as carbon black, ceramic fiber and fullerenes are widely used because of their desirable properties in industrial, medical and cosmetic fields. Accordingly, these particles can be released, or as vapors, into the human environment and then can be inhaled. However, there are few data about manufactured nanoparticles on mammalian mutagenesis and carcinogenesis. In the present study, we examined the genotoxic effects of carbon black, kaolin and fullerenes by an *in vivo* mutation assay system using *gpt* delta transgenic mice. Male mice were intratracheally instilled with single doses of 0.05 or 0.2 mg per animal of nanoparticles. Twelve weeks after instillation, all three nanoparticles increased *gpt* mutant frequencies in a dose dependent manner in the left lungs. Among these, mutant frequencies induced by kaolin and fullerenes at high doses were increased by 2-fold compared with vehicle-instilled animals. On the other hand, mutant frequencies observed in the right lungs were slightly increased but not as significant as those of the left lungs. Moreover, these nanoparticles also induced DNA damages measured by Comet assay. From these observations, manufactured nanoparticles were shown to be genotoxic in *in vivo* assay systems. We are now investigating the mutational characteristics induced by nanoparticles.



P1- 009

How small aggregates must be prepared in an in vitro safety evaluation system for nanomaterials?

Atsuko Matsuoka[1], Yoshie Matsuda[1], Ryusuke Nakaoka[1], Yuji Haishima[1], Masako Yudasaka[2], Sumio Iijima[3], and Toshie Tsuchiya[1]

[1]National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

[2]AIST Nanotube Research Center, AIST, Tsukuba 305-8565, Japan

[3]Meijo University, 1-501 Shiogamaguchi, Tenpaku-ku, Nagoya 468-8502, Japan

Introduction. Recently, toxicological concerns of nanomaterials (NMs) have been increasing with their development. Therefore, we are trying to establish an in vitro screening system of NMs to evaluate their human safety. In a prior study, we found that a specific size of particles had much stronger cytotoxicity than the others. We have been trying to disperse NMs as homogeneously and small as possible in suspension for a biological test system. We, here, put a question on particle sizes to be tested.

Materials and methods. A Chinese hamster cell line CHL was used for the cytotoxicity test (the colony formation assay). Eleven sizes of polystyrene particles were obtained from Spherotec Inc. (IL, USA). As-grown single-walled carbon nanohorn (NHas) was prepared by CO_2 laser ablation of a pure graphite target containing no metal catalyst [1].

Results. We tested cytotoxicity of eleven sizes of polystyrene particles ranging from 0.1 to 9.2 μm in mean diameter utilizing CHL cells. The three smallest sizes of particles ranging from 0.1 to 0.51 μm were much less cytotoxic than the other particle sizes. Particles in a size range 2.07 to 4.45 μm showed the strongest cytotoxicity. We also prepared six kinds of suspension with NHas under different ultra-sound sonication conditions. The particle sizes of the suspensions were ranged from 2.1 to 5.1 μm in median. Cytotoxicity of the suspensions was almost the same with 50% growth inhibition mass concentrations of around 135 μg/ml (Fig. 1). The relationship between the ratio of particles ranging from 2.07 to 4.45 μm in the particle size distribution and their cytotoxicity will be discussed.

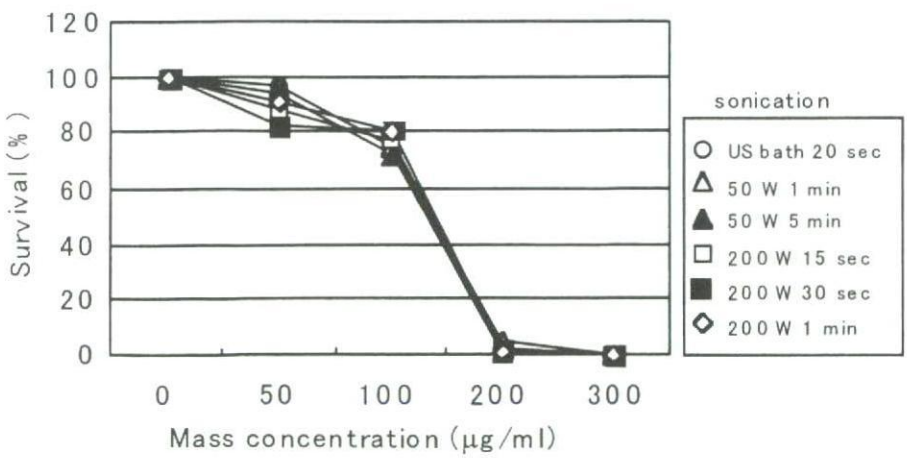


Figure 1 Cytotoxicity of NHas dispersed in the culture medium under different sonication conditions

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P1- 010

Imaging of biodistribution of organic- / inorganic- particles in mice

Shigeaki Abe[1], Chika Koyama[1], Tsukasa Akasaka[1], Motohiro Uo[1], Yoshinori Kuboki[1,2], and Fumio Watari[1]

[1]Graduate School of Dental Medicine, Hokkaido University, Sapporo 060-8586, Japan

[2]Koken Bioscience Institute, Tokyo 115-0051, Japan

Introduction: Recently, micro/nano-sized organic- / inorganic- materials have received much attention in view of biocompatibility and/or nanotoxicity. To investigate the compatibility, it is important to determine the biodistribution of the materials. In this study, we determined the distribution of administered organic- / inorganic- particles in mice.

Materials and Methods: Male mice (Jcl:ICR) were used at ages from 8 to 12 weeks. Several inorganic particles were dispersed into normal saline, respectively. The concentration was adjusted to 10 mg/mL. 0.6 mL of the dispersion was injected into the tail veins of mice. Their organs were excised at several post injection time (from 0 hr. to 4 weeks). The specimens of excised organs were subjected to elemental distribution analysis using X-ray scanning analytical microscope (XGT-2000V, Horiba, Japan). The XSAM observation was carried with the incident X-rays generated from an Rh anode under conditions of 30kV and 1 mA. In the case of organic particles, the concentration was lower than that of inorganic particles. The administered particles were determined with transmission electron microscope (H-800, Hitachi, Japan) or some microscopic method.

Results & Discussion: Elemental distribution images of a whole-mouse that were administered TiO_2 particles were determined with XSAM. At the initial period, fluorescent X-ray of Ti was only obtained in the lung tissue. The fluorescence was decreased with post injection time, and then disappeared within 24h. Contrastively, the fluorescence from liver was increased with the time. It was suggested that the TiO_2 particles left from lung quickly by blood circulation then arrived at liver. This phenomena was clearly different from a typical small molecule. To determine more details of the distribution, mice's organs were excised at each post injection time. The results suggested that each administered material had the individual distribution property and some of them showed their time-dependence. In addition, it was suggested that the distribution of particles was depended on the diameter of particles.

Conclusions: In this study, we succeeded to visualize of biodistribution of organic- / inorganic- particles in mice. The effects of elements and diameter, and the time-dependence were also determined.

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Assessment of allergic hypersensitivity to dental materials

M. HOSOKI[1], E. BANDO[1], K. ASAOKA[2], M. KITAMURA[1], H. TAKEUCHI[1], and K. NISHIGAWA[1]

[1]Department of Fixed Prosthodontics, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho Tokushima, Tokushima, 770-8504, JAPAN

[2] Department of Biomaterials and Bioengineering, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho Tokushima, Tokushima, 770-8504, JAPAN

Objectives: Many kinds of metallic elements are utilized as dental prostheses in a mouth. These materials may cause several allergic hypersensitivity reactions to patients[1]. Allergic reactions are most likely tissue reactions to dental materials. They appear not only in an oral cavity but also on hands, feet or a whole body. Ionic metal release can be a cause of allergenic reactions; however micro-particles of the corrosion products and/or ionic metal hydroxides/oxides are suspected to allergen. The purpose of this study is to review our clinical surveillance of the dental allergic hypersensitivity.

Methods: We studied 212 patients who visited Tokushima University Dental Hospital in the period from July 2000 to June 2005 with suspected dental metal allergy. To identify the allergen, a skin patch-test was performed.

Results: The results were as follows; pustulosis palmaris et plantaris (42.9%) (Fig. 1), liphen planus (11.3%) (Fig. 2), erosion of oral mucosa or gingivitis (9.9%), dermatitis (8.5%) (Fig. 3), other symptoms (27.4%). 148 of 212 suspected patients (69.8%) exhibited positive reaction to the patch-test.

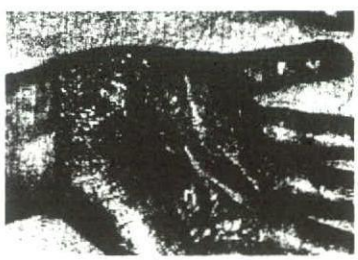

Fig. 1 pustulosis palmaris et plantaris

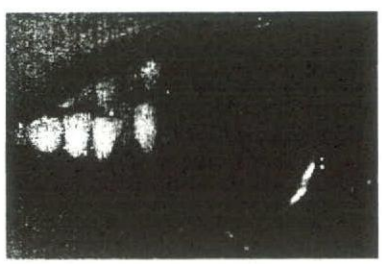

Fig. 2 liphen planus

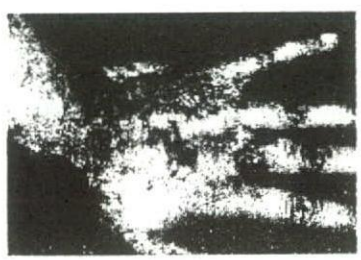

Fig. 3 dermatitis

Conclusion: These results indicated that dentists and dental researchers were concerned about the possible allergenic and/or carcinogenic potential of the dental alloys. Reactions of mucous membrane with metal ions/fine metal particles will become clear in the future.

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P1- 012

Nano-micro Mapping Analysis of Human Bone Integrated with Orthodontic Midpalatal Implant

Masaru Murata[1], Toshiyuki Akazawa[2], Toshihiro Yuasa[1], Miki Okayama[1], Junichi Tazaki[1], Takao Hanawa[3], Makoto Arisue[1] and Itaru Mizoguchi[1]

[1] Health Sciences University of Hokkaido, 1757 Kanazawa Tobetsu-cho 061-0293, Japan

[2] Hokkaido Industrial Research Institute, Nishi-11 Kita-19 Kita-ku, Sapporo 060-0819, Japan

[3] Tokyo Medical and Dental University, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan

< Introduction > Midpalatal implant was developed as a new orthodontic implant system in 1996, Switzerland [1]. In our clinic, 43patients (34 females and 9 males, mean age: 21 years 10 months) received one screw-type endosseous implant each (fixture lengths: 4 or 6mm, diameter: 3.3mm; Orthosystem®, Institute Straumann, Switzerland) for maximum orthodontic anchorage [2]. The success rate of the initial midpalatal implants (length: 6mm) was 96.3% (26/27), while the success rate of the length of 4mm was 75.0% (12/16). About 2-3 years after the implantation, the midpalatal implant should be removed surgically during orthodontic treatments. Until now, there is no basic report involved in the intensity of human bone that supported for the midpalatal implant. The aim of this study is to analyze the intensity of functional bone after dynamic treatments using the midpalatal implant.

< Case > Patient: 18 years old, male. Diagnosis: Malocclusion.

Orthodontic implant system: Pure titanium fixture, lengths: 4 mm, diameter: 3.3mm; Orthosystem®

Duration used as orthodontic anchorage: 3 years.

Preparation for removed sample: Resin-embedded section stained by toluidine blue.

Analysis machine: D8 DISCOVER with GADDS TXS®, BRUKER CORP., TOKYO.

Measurement of mapping: Angle of X ray-incidence; 13degrees, interval(X,Y); 50 μ m, score; 12(X)x15(Y)=180, analyzed peak; Hap (002), time; 20min/sample.

< Results > The map revealed functionally graded distribution of Hap (002) intensity pilots (high;37,000, low;900), and a cortical bone-like structure (lamella, osteon) was found histologically in the supportive bone of the mapping area.

Literature References

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P1- 014

Cell adhesion to CNT coated silicone rubber

Makoto Matsuoka[1], Tsukasa Akasaka[1], Takeshi Hashimoto[2],
Yasunori Totsuka[1], Fumio Watari[1]

[1]Graduate School of Dental Medicine, Hokkaido University, Sapporo, Hokkaido, Japan

[2]Meijo Nano Carbon Co., LTD., Nagoya, Aichi, Japan

Introduction:

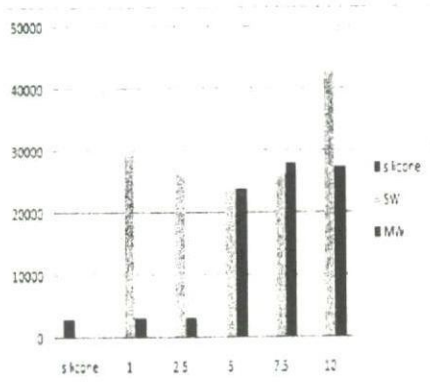
Silicone implants are used worldwide, because of its flexibility and inert character. While silicone implants hardly cause serious infection, they have little cellular adhesiveness and therefore have problems such as the formation of the fibrous capsule with the surrounding tissue.

As for the carbon nanotubes (CNTs) with unique character, various applications are expected in the biofield. In this study, we focused on biocompatibility of CNTs and tried to improve cellular adhesiveness by coating a silicone rubber with CNTs.

Materials and Methods:

SWCNTs and MWCNTs were dispersed in ethanol by sonication, and the resulting dispersion was poured onto silicon rubber sheet and dried more than three hours. Five different substrates in quantity of CNTs were prepared in both CNTs type. Surface structures were observed using scanning electron micrograph (SEM). Surface roughness and contact angles were measured. Protein adsorptions to the substrates were also evaluated. And then, Saos2 cells were cultured on each substrate for 6days. After cultivation, cell proliferation was evaluated by counting numbers of cells adhered to each substrate. And cell morphology was observed with SEM.

Results and Discussion:



SEM observation of the substrates showed that CNTs formed densely packed meshwork structure on silicone. Surface roughness and contact angles were almost not different in each substrate. In the protein adsorption, there was certain degree of difference between both CNTs and silicone only. After 6 days cultivation, cells had scarcely multiplied on the non-treated silicone. By contrast, a large number of cells were proliferated on SWCNT substrates. In the case of MWCNTs, with increasing quantity of CNTs on the substrate, cell numbers increased. SEM observations showed the similar results.

These findings indicate that CNTs improve cellular adherence to and proliferation on silicone rubber. Therefore, CNT coated silicone would become a useful material for tissue implant.

Cell Culture on Imogolite Scaffold

Kosuke Ishikawa[1], ▫Masayuki Kaga[1], Tsukasa Akasaka[2],
Yasutaka Yawaka[1], Masaya Suzuki[3], Fumio Watari[2]

[1] Department of Dentistry for Children and Disabled Person, Division of Oral Functional Science, Hokkaido University, Sapporo, Japan
[2] Department of Biomedical, Dental Materials and Engineering,Graduate School of Dental Medicine, Hokkaido University, Sapporo, Japan
[3] AIST, Institute for Geo-Resources and Environment, Tsukuba, Japan

Introduction:

Imogolite is a rare nanotube-shaped mineral with a chemical composition of $[Al_2O_3SiO_42H_2O]_n$ in nature. Until imogolite has been pushed forward in a pedological field, but imogolite attracts attention as the nano-material which there is in nature originally in the late years. Because imogolite has high surface area by peculiar shape, hydrophilicity, and high adsorption characteristics, various industry applications are expected. However, imogolite has not been applied for bio-applications. In this study, imogolite coated dishes were prepared and their properties on culturing of osteoblast like cells were evaluated.

Materials and Methods:

To prepare imogolite coated dishes, aliquot of imogolite dispersion was pour into a cell culture dish and dried. The surface characteristics of the resulting dishes were evaluated by SEM observation, wettability, and surface roughness. Then, to evaluate biocompatibility, human osteoblast like cells (Saos2) were cultured on the dishes and then cell activity, proliferation, and cell adhesion were estimated.

Results and Discussion:

Imogolite was homogeneously coated on cell culture dish (Fig.1), and the cells were widely spread on the imogolite coated surface (Fig.2). The cell number of the dishes increased similar to that on cell culture dish. In addition, cell adhesion to imogolite coated dish was higher than that to cell culture dish.

As a result, imogolite did not show the toxicity in the short term *in vitro*, and the cells proliferated well. It is supposed that imogolite could be a biocompatibility material. Therefore, imogolite could be effective biomaterials for the cell adhesion /cell proliferation.

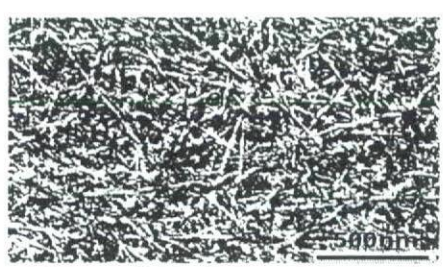


Fig. 1 SEM micrograph of imogolite scaffold

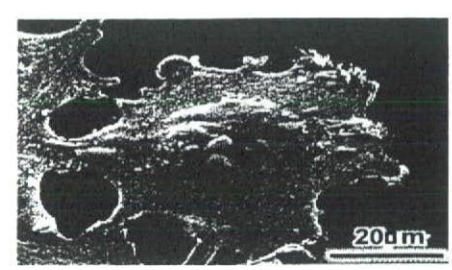


Fig.2 SEM image of Saos2 attached on imogolite coated dish.

Evaluation of Waveform-like Pattern of Cell Proliferations on Self-Assembled Monolayers with a Series of Surface Composition Changes

I. Hirata and M. Okazaki

Department of Biomaterials Science, Graduate School of Biomedical Sciences,
Hiroshima University, Hiroshima 734-8553, JAPAN

Introduction:

Vital reactions, such as tissue adhesion, inflammation, and immunoreaction, basically arise from the reaction between the material surface and biomolecules. It is therefore necessary to examine surface conditions carefully with consideration of surface factors, such as surface functional group ratio, phase separation, mobility, and surface morphology.

We made mixed self-assembled monolayers (mixed SAMs) with various ratios of amino, hydroxyl, carboxyl, and methyl surface head groups and report the relationship between the surface composition and water contact angle, as a series of the surface composition changes induces cell growth and differentiation of the growth pattern of each cell under the same surface conditions.

Materials and Methods:

Gold substrates were cleaned by immersion in piranha solution and washed by ultra pure water. Then, 1 mmol/L of 11-amino-1-undecanethiol (NH_2), 11-mercapto-1-undecanol (OH), 11-mercapto-1-undecanoic acid (COOH), and 1-dodecane-thiol (CH_3) solutions were prepared in ethanol. These alkanethiol solutions were mixed at various ratios. The gold plates were immersed in these solutions for 24 hours, and the mixed SAMs were formed on the surface of the gold substrates. Wettability and surface composition of the mixed SAMs were measured by static contact angle measurement apparatus and X-ray photoelectron spectroscopy. 4 kinds of cells (A1, C3H10T1/2-clone8, UVB6-2.1A, and UV□2) were cultured on the mixed SAMs for 3 days and evaluated between surface composition and cell proliferation.

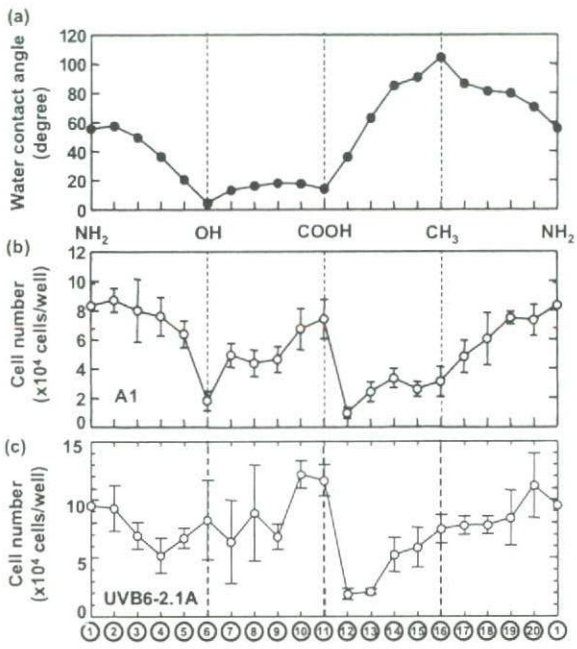


Figure 1. (a) water contact angles of mixed SAMs, (b) cell number of A1 for 3 day incubation, (c) cell number of UV6-2.1A for 3 day incubation. Abscissa axis is mol ratio between NH_2 and OH (①~⑥), between OH and COOH (⑥~⑪), between OH and COOH (⑪~⑯), and between CH_3 and NH_2 (⑯~①).

Results and Discussion:

The relationship between wettability and surface composition are shown in figure 1a. For this result, we were able to evaluate the waveform-like patterns between surface composition and cell proliferation strictly (figure 1b,1c). The effect of the surface functional groups on cell proliferation using 4 cells was examined, and it was able to be shown that the waveform-like pattern of cell proliferation for the surface composition change differed for each cell.

Biocompatibility of binder-free multi-walled carbon nanotube blocks cross-linked by de-fluorination against subcutaneous tissue of rats *in vivo*

Yoshinori Sato[1], Atsuro Yokoyama[2], Takao Kasai[2], Shin-ichi Ogino[1], Naoki Sashida[1], Shinji Hashiguchi[3], Balachandran Jeyadevan[3], Kazuyuki Tohji[3]

[1] Graduate School of Environmental Studies, Tohoku University, Sendai, 980-8579, Japan.

[2] Graduate School of Dental Medicine, Hokkaido University, Sapporo, 060-8586 Japan.

[3] Stella Chemifa Co., 1-41, Rinkai-cho, Izumiotsu, Osaka, 595-0075, Japan.

Since the excellent antithrombotic property of graphite was discovered in 1963, biocompatible artificial organs or internal bone plates have been developed using carbon materials such as low temperature isotropic carbon (LIT) in high density isotropic carbon materials, carbon fiber-reinforced carbon composites (C/C) and polymer/carbon fiber composites. In particular, C/C have been used as an alternative hard tissue material of bone or dental root, which have been generated from carbon fibers (CFs) and resins by a combination of resin impregnation and hot press methods. Recently, basic biomedical research concerning drug delivery and the use of scaffolds and catheters has been carried out using a new type of carbon allotrope comprising carbon nanotubes (CNTs). We have investigated the use of alternative artificial hard tissue materials comprising CNT composites. As CNTs possess low density, high mechanical strength and protein adsorption properties, artificial hard tissue alternative materials comprising CNTs can potentially be utilized as porous and strong 3-dimensional materials composed of 1-dimensional CNTs that offer advantages in comparison with the use of traditional C/C materials. We have produced large-sized binder-free multi-walled carbon nanotube (MWCNT) blocks from fluorinated MWCNTs using thermal heating and a compression method *in vacuo* (the blocks are referred to as "de-F-MWCNT blocks") [1].

Generally, use of the "implant test *in vivo*", the most important and basic toxic evaluation method, is employed to examine material responses to tissue *in vivo* as well as cellular responsive reactions *in vitro*. Here, we report on the tissue response in subcutaneous tissue and evaluate the biocompatibility of binder-free de-F-MWCNT blocks using rats *in vivo*. MWCNT/resin blocks carbonized with 50wt% phenol resin were examined, with poly(methyl methacrylate) (PMMA) and Ni being used as a negative and positive controls, respectively.

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Development of multi walled carbon nanotubes coated collagen for cell culturing

Michiko Terada[1], Motohiro Uo[2], Yoshimasa Kitagawa[2] and Fumio Watari[2]

[1]Department of Oral Pathobiological Science, Graduate School of Dental Medicine, Hokkaido University, Japan

[2]Department of Biomedical, Dental Materials and Engineering, Division of Oral Health Science, Graduate School of Dental Medicine, Hokkaido University, Japan

Introduction

Carbon nanotubes (CNTs) are one of the most interesting nanomaterials because of mechanical, chemical and electrical properties. Recently, the biocompatibility of CNTs has been reported [1]. In this study, multi walled carbon nanotubes (MWCNTs) coated cell culture dishes were prepared and their properties on all culturing were evaluated.

Materials and methods

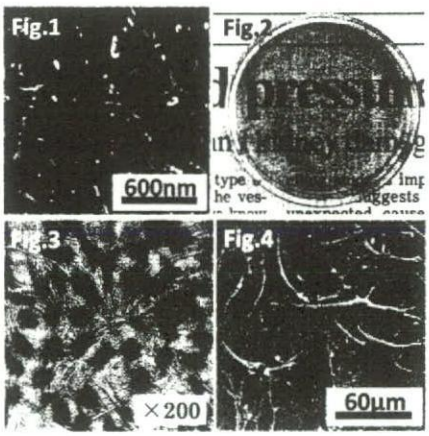
Carboxylated MWCNTs were dispersed in aqueous sodium cholate solution [2]. The collagen-coated dish (IWAKI&CO., LTD, Japan) was treated the MWCNTs suspension and MWCNTs coated dish was prepared. MC3T3-E1 cells were cultured on the MWCNT-coated dish and their cell viability, proliferation and adhesion were estimated.

Results

MWCNT-coated dish surface was homogeneously coated by MWCNTs(Fig.1) and had good transparency (Fig.2). The cell viability and proliferation on the MWCNT-coated dish was similar to those on the collagen-coated dish. Fig.3 showed optical microscopic image and Fig.4 showed SEM image of cells on the MWCNT-coated dish. The cells spread widely and attached on the MWCNT-coated dish. The cell adhesion on MWCNT-coated dish was much higher than that on the collagen-coated dish.

Conclusion

MWCNT-coated dish showed the good biocompatibility and high cell adhesion. Therefore, MWCNT-coated dish which is developed in this study would be applicable as a new cell culture substrate.



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P1-019

T Low-voltage and high-voltage TEM observations on CNT of rat in vivo

N. Sakaguchi[1], F. Watari[2], A. Yokoyama[2], Y. Nodasaka[2] and H. Ichinose[3]

[1] Center for Advanced Research of Energy Conversion Materials, Hokkaido University, Japan

[2] Graduate School of Dental Medicine, Hokkaido University, Japan

[3] Frontier Research System, Riken, Japan

The development of nanomaterials for Drug Delivery System (DDS) is essential for the realization of health promotion and quality of life in the aged society of the 21st century. Efforts have been extensively done for the development of nanotechnology to exploit the new functional materials. However it is natural that both merits and demerits may occur by the enhancement effects due to nanosizing of the materials. The terminology "nanotoxicology" appeared a few years ago.

Carbon nanotubes (CNT) have attracted up to now the attentions to the application in the electronic and chemical fields such as field emission electron sources, quantum device materials, and hydrogen storage materials; however, the application for the biomedical field has been very rare. There are arguments that the CNT may have the serious toxicity due to its acicular or fibrous particle shape, associated with lung carcinogenicity of asbestos. Thus, the CNT is regarded as one of the most typical examples of "nanotoxicology" due to its morphology.

In the present study, we focused on an optimal condition for morphology and atomic structure analysis of CNTs in vivo by transmission electron microscopy (TEM). Either low-voltage or high-voltage TEMs were chosen for the high-contrast or high-resolution imaging of the CNTs respectively. We will also discuss the degradation mechanism of the CNTs of rats in vivo observed by the high-resolution TEM.

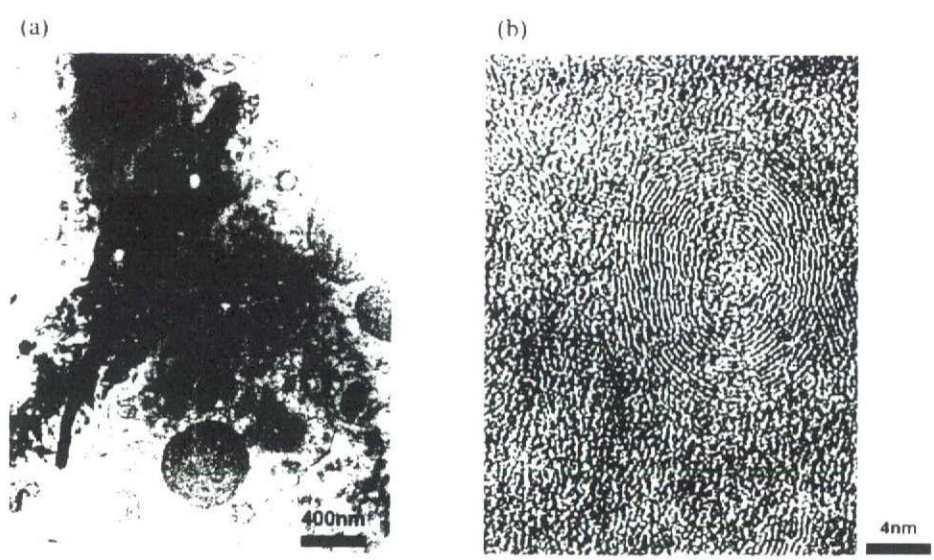

Fig.1 (a) Low-voltage and (b) high-voltage TEM images of CNTs in vivo.

Carbon nanotubes incorporate into cell structure; a novel pathway for nanotubes detoxification in plants.

Maged Fouad, Noritada kaji, Mohamad Jabasini, Manabu Tokeshi and Yoshinobu Baba

Department of Applied Chemistry, Graduate School of Engineering, Nagoya University, Nagoya 464,8603 Japan

Since, their discovery, carbon nanotubes (CNTs) have been eminent members of the nanomaterial family. Because of their unique physical, chemical and mechanical properties, they are widely predicted and regarded as new potential materials to bring enormous benefits in cell biology studies. Undoubtedly, the first step of this approach has to be understanding and tracking the basic behavior of CNTs inside living cells. In a number of studies, CNTs have been demonstrated as new carrier systems for the delivery of DNA, proteins and therapeutic molecules into living cells [1]. However, post-transfection behavior of CNTs inside cells has not been demonstrated previously. This study aims at evaluating CNT toxicity and tracking their post-transfection behavior. CSCNT was observed to slightly increase the viability of cultured Arabidopsis cells in suspension culture (Fig.1). One possible reason is the antioxidant property of carbon nanotubes in solution [2]. Also, activated carbon has been proven to stimulate cell growth in suspension culture through adsorption of polyphenolic toxic compounds secreted by the plant cells [3]. Utilizing the plant cell model, we have confirmed that plant cells, differentiating into tracheary elements (TEs) (Fig. 2), incorporate cup-stacked carbon nanotubes into cell skeleton via oxidative cross-linking of monolignols to nanotubes surface during lignin biosynthesis. Result were confirmed through AFM monitoring of DHP (in vitro lignin model compound) in presence and in absence of carbon nanotubes. DHP was observed to attach to CNT in a similar fashion to its deposition into the secondary cell wall of the plant cell (Fig. 3). This finding highlights the fate of CNTs inside plant cells and provides an example on how the cell can handle internalized nanomaterials.

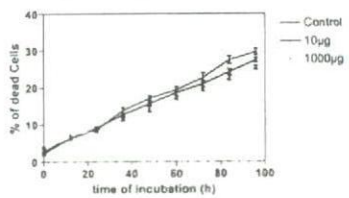

Fig. 1 CNT Toxicity testing

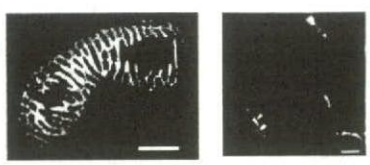

Fig. 2 CNT (red) inside TEs

Fig. 3 CNT attachement to DHP

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Human Osteosarcoma Cell Adhesion onto Carbon Nanotube Sheets

Tsukasa Akasaka[1], Atsuro Yokoyama[1], Makoto Matsuoka[1], Takeshi Hashimoto[2], Shigeaki Abe[1], Motohiro Uo[1], Fumio Watari[1]

[1]Graduate School of Dental Medicine, Hokkaido University, Sapporo, Hokkaido, Japan

[2]Meijo Nano Carbon Co., LTD., Nagoya, Aichi, Japan

Introduction:

Carbon nanotubes (CNTs) have been attracting considerable attention because of their unique physical properties and potential for a variety of applications. Our recent studies employing *in vitro* experiments showed their excellent properties of cell adhesion, proliferation, and protein adsorption as scaffolds for cell culture. However, there are only a few data about the cell adhesion of osteoblasts onto CNT sheets. In this study, we investigated cell adhesion of human osteosarcoma cell (SaOs2) onto CNT sheets.

Materials and Methods:

The purified SWNTs employed were purchased from Meijo Nano Carbon Co., Ltd. (Nagoya, Japan); and the MWNTs used were purchased from NanoLab Inc. (Brighton, MA). CNTs sheets were prepared by our previous methods. To estimate the ability of cell adhesion onto CNTs sheet, SaOs2 was seeded in DMEM containing 10% FBS and incubated for 1h, 3 h, and 6 h. For SEM observation, the sheets were fixed with a solution of 2.5% glutaraldehyde and dehydrated following critical-point drying at 37°C. Cell adhesion was evaluated by counting the number of cells attached to each sheet in SEM images.

Results and Discussion:

Figure 1 shows a large number of cells on CNT sheets were observed after 1h incubation. On contrast, a number of cells on cell culture dish were increased with increasing time. In particular, CNT sheet exhibited faster adhesion than that of cell culture dish. In the case of MWNT sheet, a number of cells were higher than that of other substances for all incubation time. These results shows MWNTs have the ability of both faster adhesion and higher-capacitance of cell adhesion. The higher adhesion onto MWNT sheet could be caused by its rough surface and protein adsorption property. Thus, CNT sheets could become a useful material for cell culture scaffold.

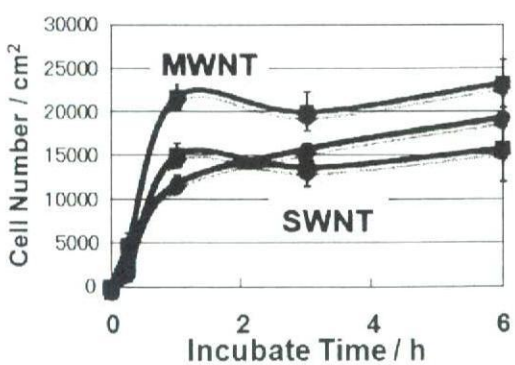

Fig 1. Cell adhesion assay onto CNT sheets

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Application of Nano-crystal CO_3Ap as Hard Tissue Scaffold Biomaterials

M. Okazaki and I. Hirata

Department of Biomaterials Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8553, JAPAN

Biocompatible and biodegradable scaffold biomaterials are expected for hard tissue regeneration. To develop a new functional scaffold biomaterial reinforced with a frame, nano-crystal CO_3Ap was synthesized and mixed with neutralized collagen gel, and the CO_3Ap-collagen mixtures were lyophilized into sponges in a porous HAp-frame ring. X-ray diffraction (Fig. 1) and FT-IR analyses together with chemical analysis indicated that synthesized CO_3Ap had crystallinity and a chemical composition similar to bone. SEM observation showed that the CO_3Ap-collagen sponge had a suitable pore size for cell invasion (Fig. 2). In proliferation and differentiation experiments with osteoblasts, ALP and OPN activity was clearly detected. When these sponge-frame complexes with rh-BMP2 were implanted beneath the periosteum cranii of rats, sufficient new bone was created at the surface of the periosteum cranii after 4 wks' implantation (Fig. 3). Furthermore, when the CO_3Ap-collagen sponge containing SVVYGLR peptide was implanted as a graft into a tissue defect created in rat tibia, the migration of numerous vascular endothelial cells as well as prominent angiogenesis inside the graft could be detected after 1 week. These CO_3Ap-collagen sponges with highly functional modification are expected to be used as hard tissue scaffold biomaterials for the therapeutic purpose of rapid cure, especially for patients with lower regeneration ability.

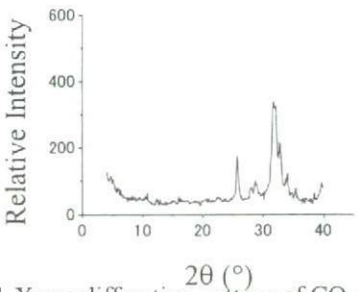

Fig. 1 X-ray diffraction pattern of CO_3Ap.

Fig. 2 SEM photo of CO_3Ap-collagen sponge.

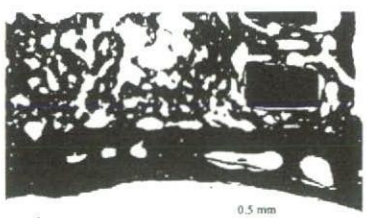

Fig. 3 Hematoxylin-eosin staining of newly created bone with CO_3Ap-collagen sponge reinforced with a HAp frame implanted onto the periosteum cranii of rats.

Microstructure Design of Biomimetic and Functionally Graded Hydroxyapatite by Calcination and Partial Dissolution-Precipitation Methods

Toshiyuki Akazawa[1], Masaru Murata[2], Junichi Tazaki[2], Katsuo Nakamura[1]
Jun Hino[2], Masaya Yamamoto[3] and Yasuhiko Tabata[3]

[1] Hokkaido Industrial Research Institute, Nishi-11 Kita-19 Kita-ku, Sapporo 060-0819, Japan
[2] Health Sciences University of Hokkaido, 1757 Kanazawa Tobetsu-cho 061-0293, Japan
[3] Kyoto University, 53 Kawahara-cho, Seigoin, Sakyo-ku, Kyoto 606-8507, Japan

Hydroxyapatite (HAp) has been applied as bioactive and substituted materials for hard tissues in dental and medical fields because of excellent biocompatibility and osteoconduction. To arrange the required capability of HAp products for the biomaterials early incorporated into bio-metabolic system, we tried to develop biomimetic and functionally graded HAp (fg-HAp) ceramics in which grain size and crystallinity of HAp gradually change from surface regions to bulk parts [1]. Spongy bovine-femur-bone was calcined at 1073-1473K for 24h to obtain crystalline HAp (b-HAp) ceramics. The spongy b-HAp was partially dissolved into HNO_3 solution by the different ways, such as stirring for hours and supersonic treatment for minutes. After the partial dissolution, HAp nano-crystals were precipitated on macro-pores and micro-pores of b-HAp by adding NH_3 solution to form fg-HAp ceramics. The crystallinity and microstructure of fg-HAp can be designed and controlled by the calcination and partial dissolution-precipitation conditions. The pore structure of fg-HAp was classified into a macro-pore (100-800μm) originating from spongy bone and a micro-pore (10-160nm). The fg-HAp ceramics were implanted into the subcutaneous tissues of the back region in rats. At 4 weeks after the implantation, body fluid extensively permeated into the bulk regions of HAp through the micro-pores of the ceramics. In the macro-pores, many multinucleated giant cell and fibroblast were observed. Surface- and bulk-degradations of the HAp proceeded, so that a total size of the HAp ceramics remarkably decreased. Also, microcracks in bulk regions of the ceramics formed by the partial dissolution with HNO_3 under stirring or supersonic treatment will result in auto-degradation and body fluid permeation. The fg-HAp ceramics loaded with ^{125}I-labeled recombinant human bone morphogenetic protein-2 (rhBMP-2) were implanted into the back subcutis of mice. Even at 2 weeks, the retention percentage of rhBMP-2 was about 60%, suggesting that the fg-HAp ceramics are osteoinductive scaffold [2]. Accordingly, since the fg-HAp has not only degradation-absorption ability due to specific microstructure but also good rhBMP-2-adsorption-release characteristics, the rhBMP-2/fg-HAp may be one of osteoinductive and absorbable bioceramics related to bone-remodeling system.

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Injectable Biphasic calcium phosphate bioceramic: the HYDROS® concept

Baroth S. [1,2], Bourges X. [1], Goyenvalle E. [3], Aguado E. [3], Daculsi G. [2]

[1] Biomatlante SAS, 44360 Vigneux de Bretagne, France,
[2] University of Nantes, INSERM U791, Faculté de chirurgie dentaire, Nantes France,
[3] LBBTO Veterinary School Nantes, France.

Introduction:

Biphasic Calcium Phosphates (BCP) were developed since 1985. BCP is a bioactive concept based on mixture of HA and ▫-TCP. The concept is determined by an optimum balance of the more stable phase of HA and more soluble ▫-TCP. The material is soluble and gradually dissolves in the body, seeding new bone formation as it releases calcium and phosphate ions into the biological medium. The main attractive feature of BCP ceramics is their ability to form a direct bond with the host bone resulting in a strong interface compared to bio inert materials. High osteogenic/osteoinductive properties of micro macro porous BCP have been recently reported [1]. BCP nano and micro particles are increasingly used in moldable, injectable bone substitutes. However, the biological behaviour of BCP micro particles can be influenced not only by chemical composition and crystallinity, but also by porosity and size of particles. The aim of the study was to assess *in vivo* the role played by a new hydrated putty bioceramics based on specific combination of nano-micro and macrosized BCP rounded particles to obtain high osteogenic and injectable bone substitute.

Material and methods:

Rounded BCP particles were obtained by using a mecano-process agglomerating calcium deficient apatite single crystals (CDA) and sintering. The BCP particles were obtained by crushing and sieving particles into specific sizes. The mixture of large microporous rounded particles (80-200 μm) and nano, micro macroporous granules (0.5 to 1 mm) with selected specific nanometer and micrometer sized particles (from 0.1 to several μm) and demineralized water provided with a high hydrophilic paste. It formed a granular putty gel with gamma sterilization (25 K grays), ready to use in syringes (Hydros® Biomatlante France).

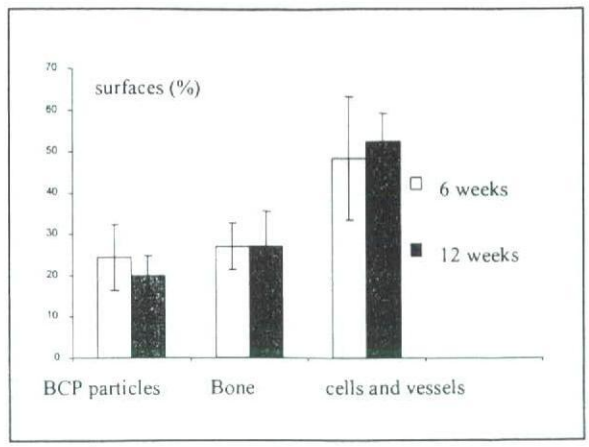
Six New Zealand rabbits were used in respect to the animal experimentation good practice. Bilateral femoral epiphysis bone defects of 6 mm were entirely filled with Hydros (0.3 cm^3). The same amount of biomaterial was injected in the lumbar muscles. After 6 and 12 weeks, the explants were fixed in a neutral formalin solution, dehydrated and embedded in GMA. Micro CT (Skyscan 1072) was performed on the samples. Histological sections of 7 μm were stained using Movat's pentachrome. Thicker sections made using a diamond saw microtome were observed by polarized light microscopy. Cross-sectioned blocks were also observed by SEM using BSE and processed by image analysis. Bone ingrowth and BCP degradation/resorption were compared using variance analysis for assessment of the statistical significance of results, Fisher's procedure, ANOVA (significance level $p<0.05$).

Results:

The putty gel obtained have a high density (d=1.5) without macropore or spaces between the larger particles. However, the putty is highly hydrophilic and permeable to biological fluids. The samples were easy to handle, fitted perfectly to the bone defect and exhibited haemostatic properties. No sign of clinical rejection was noticed in spite of high active cell resorption potential of the nano- and micro-sized particles contents.

In muscles, no fibrous encapsulation was observed, degradation of the smaller particles was noticed by macrophages and giant cells. At 12 weeks, more of 75% of BCP had resorbed with 90% of the smaller particles being resorbed In femoral defects, the same kinetics of resorption than in muscular area was observed and measured (table 1).

Table 1: Image analysis calculations of residual BCP particles, bone ingrowth and non mineralized tissue and cellular content, after 6 and 12 weeks.



The larger BCP particles remaining were surrounded by bone. Newly-formed bone was observed both at 6 and 12 weeks. Numerous bony nodules and bone trabeculae were observed, at 12 weeks bone osteoconduction on large particles confirm the scaffold effect of this type of granules [2]. Previous studies have demonstrated that small particles size, involved high macrophagous activity and then act as a booster for osteogenic cell differenciation [3] providing with abundant bone ingrowth.

Micro CT demonstrated bone evolution by remodelling and architectural organisation from 6 to 12 weeks.

Conclusion:

Combination of hydratation of selected classes of nano-micro sized particles and larger BCP granules provided favourable rheological properties for injectable calcium phosphate bioceramic formulations. This novel injectable bone substitute presents a high density (no macropores or spaces between the granules) limiting its clinical application to the filling of small bone cavities as in dental and maxillofacial surgery or for filling vertebral body in vertebroplasty. However, the small sized particles content promotes an active cellular resorption process with specific macrophagous cells (mono and multinuclear cells), followed by a promotion of angiogenesis and osteogenesis with enhanced osteoconduction by the largest BCP particles.

Acknowledgement:

We thank P. Pilet (Microscopy SC3M department), B. Fellah, and F. Moreau (Biomatlante SAS France). This work was supported by National Agency for Research ANR BioRimp 2005.

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