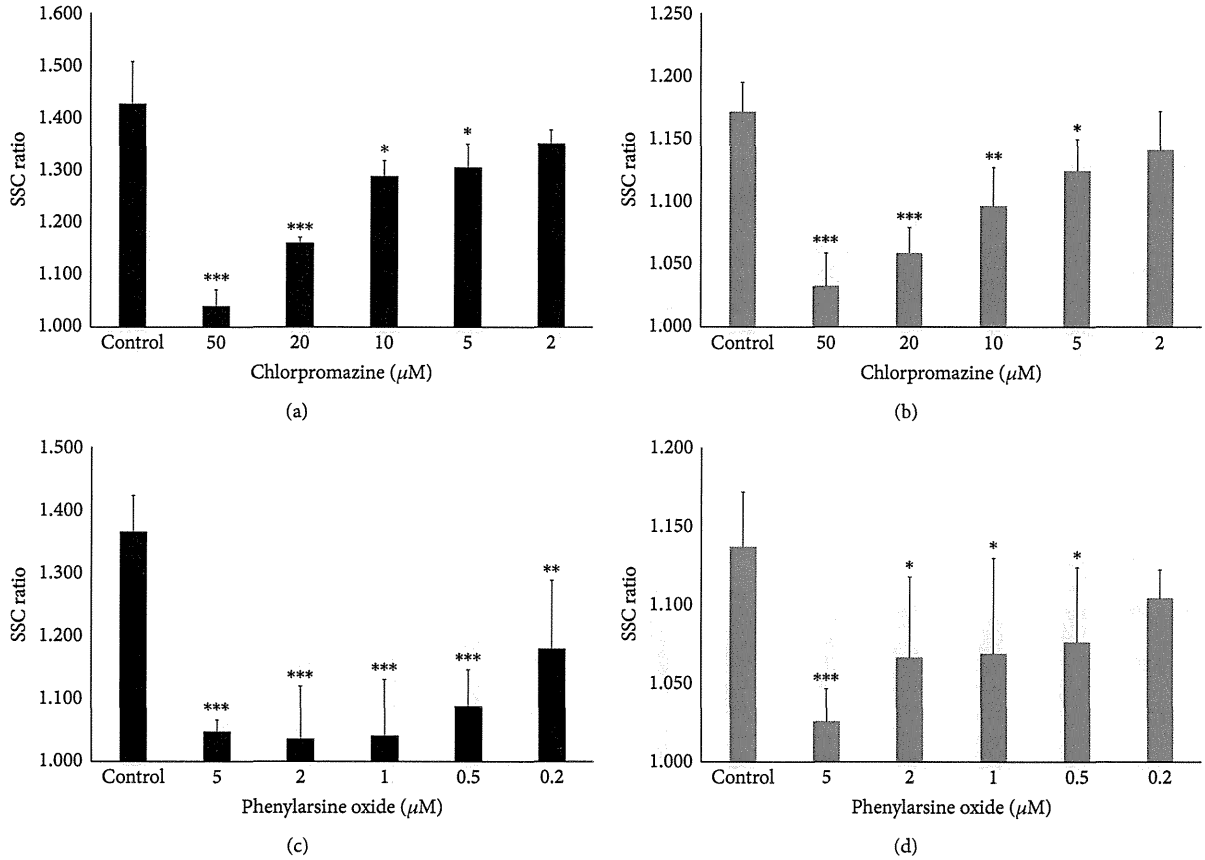

FIGURE 3: Effect of clathrin-mediated endocytosis inhibitors on cellular uptake of MWNT-7. The SSC ratios of (a) BEAS-2B cells and (b) MESO-1 cells pretreated with various concentrations of chlorpromazine are shown. The SSC ratios of (c) BEAS-2B cells and (d) MESO-1 cells pretreated with various concentrations of phenylarsine oxide are shown. The cells were compared with control cells pretreated with inhibitor solvent. Mean ± SD. $n = 4$ or 6, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

(Met-5A) [39]. However, although they showed transmission electron microscopy (TEM) images of A549 alveolar epithelial cell lines to conclude that the cells do not internalize CNTs, no TEM images for Met-5A were presented, and optical microscope images of low magnification (×20) were only shown. Lindberg et al. reported genotoxicity of MWCNTs based on TEM images showing that the Met-5A cells internalize CNTs [40]. Moreover, our time-lapse data clearly and directly indicate that HBECs and HMCs endocytose MWCNTs actively (Movies S1 and S2) (see Supplementary Material available online at http://dx.doi.org/10.1155/2015/793186). We also have already reported that the BEAS-2B cell line derived from human bronchial cells and MESO-1 cells derived from human mesothelioma cells internalized some MWCNTs [28, 36]. Therefore, we used inhibitors of endocytosis, to clarify the internalization mechanism of CNT further using BEAS-2B cells and MESO-1 cells rather than HBECs and HMCs, respectively.

We investigated the mechanism of CNT uptake using inhibitors for three endocytosis pathways (clathrin-mediated, caveolae-mediated, and macropinocytosis), with the SSC ratio as an index. We have already shown that SSC ratio increases concentration dependently over time in cells that only internalized CNTs [28]. The SSC ratios of the control, which was not pretreated by inhibitors in BEAS-2B and MESO-1 cells, were 1.355–1.426 and 1.137–1.258 in 2 h, respectively. It was observed that the SD of the SSC ratios tended to increase with cell passage number, likely because we analyzed under sixteen passages for both cell lines. Therefore, few statistically significant differences were noted when we assayed the SSC ratios of nystatin as a caveolae-mediated endocytosis inhibitor and 5-(N-ethyl-N-isopropyl)amiloride as a macropinocytosis inhibitor.

Two clathrin-mediated endocytosis inhibitors suppressed the ratio in a concentration-dependent manner in both cell lines (Figures 3(a)–3(d)). In BEAS-2B cells, the maximum concentration of chlorpromazine (50 μM) decreased the SSC ratio to 1.039, whereas the SSC ratio with 2 μM phenylarsine oxide was 1.040. In MESO-1 cells, the lowest SSC ratios were 1.032 and 1.025 with treatment with 50 μM chlorpromazine and 5 μM phenylarsine oxide, respectively. Because the baseline SSC ratio for which the cells were not exposed to CNTs was 1.000, clathrin-mediated endocytosis seems to be the main mechanism for cellular uptake.

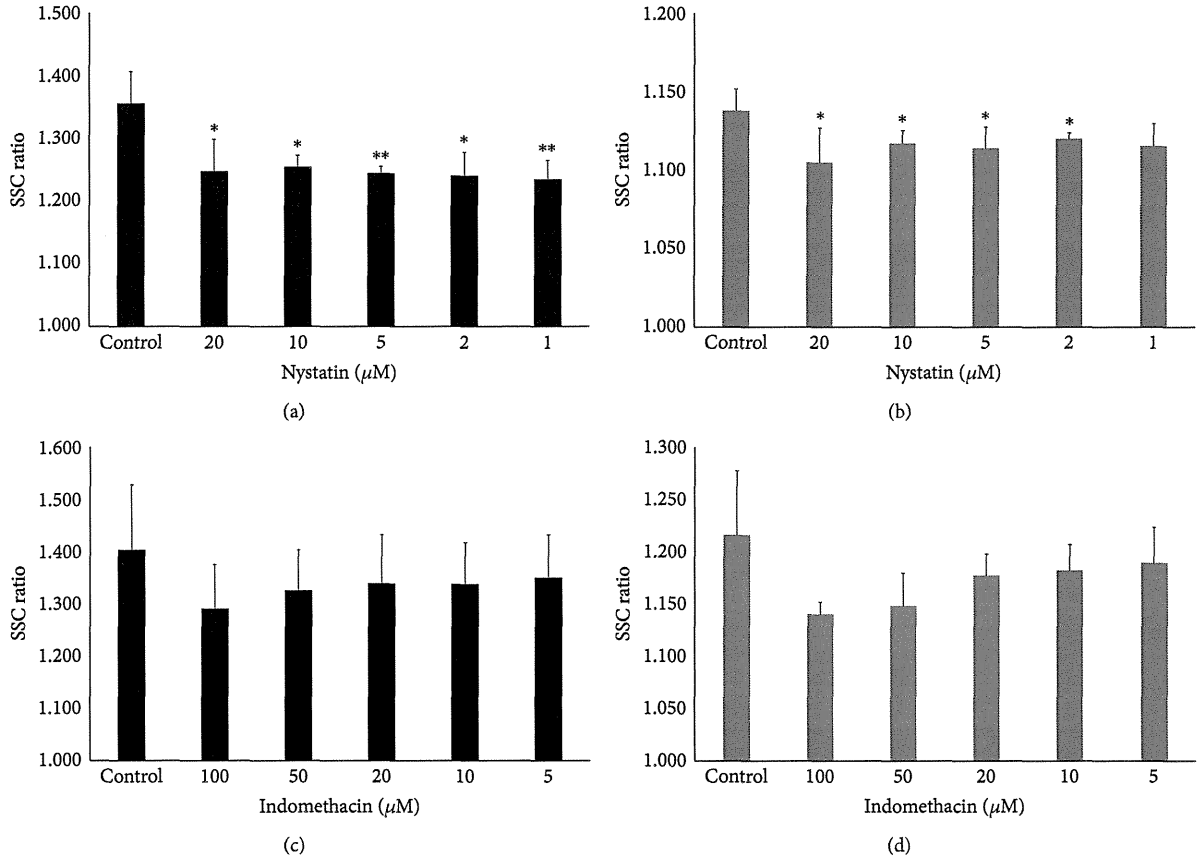

FIGURE 4: Effect of caveolae-mediated endocytosis inhibitors on cellular uptake of MWNT-7. The SSC ratios of (a) BEAS-2B cells and (b) MESO-1 cells pretreated with various concentrations of nystatin are shown. The SSC ratios of (c) BEAS-2B cells and (d) MESO-1 cells pretreated with the various concentrations of indomethacin are shown. The cells were compared with control cells pretreated with inhibitor solvent. Mean ± S.D. $n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$.

The results for caveolae-mediated endocytosis inhibitors were complicated. Nystatin decreased the SSC ratio in both cells significantly except at 1 μM in MESO-1 cells (Figures 4(a) and 4(b)). In detail, MESO-1 cells displayed a tendency for concentration dependency, whereas the inhibition did not depend on the dose in BEAS-2B cells. In contrast, although indomethacin tended to show concentration-dependent inhibition in both cell lines, there was no statistically significant difference (Figures 4(c) and 4(d)). The difference in the results may be caused by the inhibition mechanism. Nystatin disrupts caveolar function and binds to sterol in the plasma membrane [41–43]; indomethacin blocks the internalization of caveolae and the return of plasmalemmal vesicles [44, 45]. However, we considered that caveolae-mediated endocytosis pathway may partially contribute to the internalization of CNTs for the following reasons: (1) the inhibition rate of nystatin, which was not concentration-dependent, was 30.7% and was the same as the inhibition rate (27.9%) with the highest concentration of indomethacin (100 μM) in BEAS-2B cells. (2) In MESO-1 cells, both inhibitors showed a tendency for concentration-dependence, and the inhibition rate provided by indomethacin, which inhibits the essential parts of the endocytosis pathway, was higher than that by nystatin (35.2% and 23.9%, resp.). The inhibition of statin binding to the sterol may have been responsible for difference among cell types.

5-(N-Ethyl-N-isopropyl)amiloride, which inhibits the macropinocytosis pathway, seems to suppress CNT uptake in a concentration-dependent manner, although the difference did not reach significance except at 80 μM in MESO-1 cells (Figures 5(a) and 5(b)). The inhibition rates of BEAS-2B cells and MESO-1 cells were comparable at 42.7% and 56.6% at the highest concentration (80 μM). The role of macropinocytosis in CNT uptake has not been extensively studied. Hirano et al. demonstrated that macrophage receptor with collagenous structure- (MARCO-) transfected CHO-K1 cells takes up MWCNTs via membrane ruffling in a process similar to macropinocytosis [46]. They also reported that MARCO was absorbed in MWCNTs to which macrophages were exposed [47]. However, it was not clear whether macropinocytosis for CNTs occurs in nonphagocytic cells. Our results indicate that macropinocytosis plays an important role in CNTs uptake.

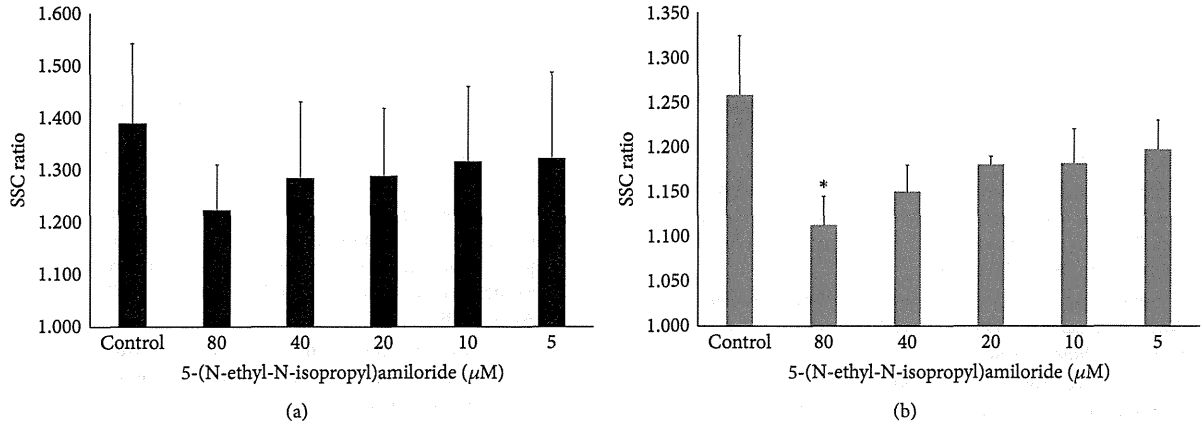

FIGURE 5: Effect of a macropinocytosis inhibitor on cellular uptake of MWNT-7 cells. The SSC ratios of (a) BEAS-2B cells and (b) MESO-1 cells pretreated with various concentrations of 5-(N-ethyl-N-isopropyl)amiloride are shown. The cells were compared with control cells pretreated with inhibitor solvent. Mean ± SD. $n = 3$, $^*P < 0.05$.

The latest information for cellular uptake of nanomaterials has been reviewed and suggests that three different mechanisms of endocytosis exist including clathrin- and caveolae-independent endocytosis, and also endocytosis depends on particle physical-chemical properties, experimental conditions, and cell type in nonphagocytic cells [48]. We consider that CNT uptake is also subject to the same influences in an interdependent manner because the total inhibition rate when independently inhibited pathways were considered together easily exceeded 100%, which means that other pathways function in a compensatory manner, even when one pathway is inhibited. Moreover, other pathways may exist because there are some reports indicating several types of clathrin- and caveolae-independent endocytosis, and the endocytic mechanism is especially unexplained in the nonphagocytic cells [49–51]. In fact, it was not possible to clarify the mechanism underlying the observed suppression of CNT uptake in BEAS-2B cells cultured in FBS-free medium [32]. That study also indicated that the degree of aggregation is an important factor but we could not clarify this issue. We measured the SSC ratio in the comparatively early stage of 2 h after CNT exposure because high concentrations of the inhibitors showed cytotoxicity. Within 2 h, a small fibrous agglomerate containing some MWCNTs was seen at the bottom of the dish. Although it seems likely that our inhibitor results reflect actual endocytosis, it is unclear whether the nonagglomerated MWCNTs observed after 2 h at the bottom in Movie S1 and Movie S2 show the same result. However, there appeared to be a common cellular uptake pattern for the MWCNTs.

In conclusion, we found that human normal bronchial epithelial cells and mesothelium cells endocytosed MWCNTs. The mechanism of endocytosis seemed to be not only one but a combination of three pathways: clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis. Although clathrin-mediated endocytosis played the most important role, other pathways may be involved to varying degrees. The cellular uptake of MWCNTs is essential for MWCNT toxicity in the context of genotoxicity. It may thus be necessary to prepare materials that are not endocytosed to develop the nanomaterials having not only useful but also hazardous properties, as we alluded to in a previous study [28]. We have already reported that both BEAS-2B and MESO-1 cells did not endocytose MWCNTs dispersed in carboxymethyl cellulose. Therefore, this and previous studies suggest that biocompatible nanomaterials can be developed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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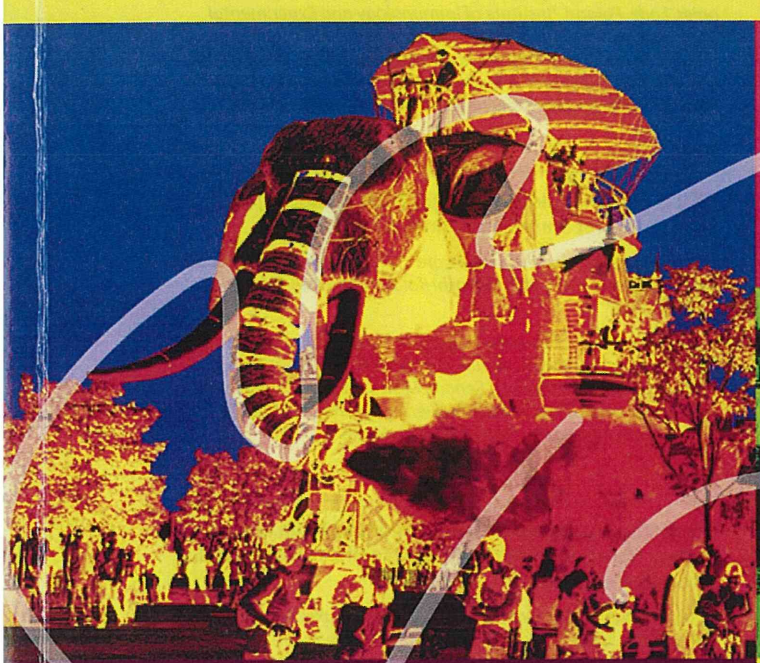

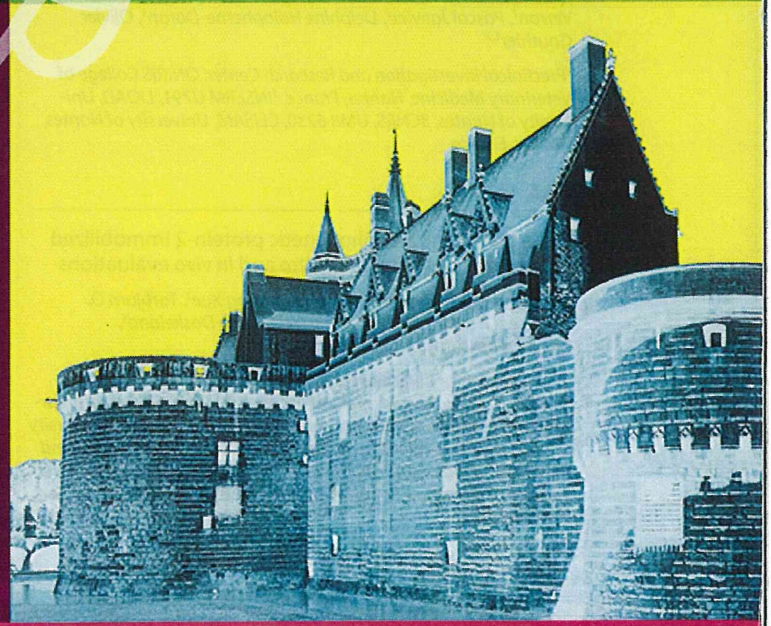

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P7 Biomaterials

P7.1

Biomaterial antibacterial efficiency with reduced level of porosity

Ingus Skadins[1], Juta Kroica[1], Ilze Salma[2], Aigars Reinis[1], Marina Sokolova[2], Natalija Berza[1]

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P7.2

Hydrogel Encapsulating Antibiotic in Osteomyelitis Prevention in Rats

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P7.3

Animal models of peri-prosthetic joint infection. Literature review and description of our model.

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P7.4

Bupivacaine-Loaded Injectable Calcium Phosphate Cement can reduce Postoperative Pain in iliac bone graft model dogs.

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P7.5

Efficacy of bone morphogenetic protein-2 immobilized on copolymer scaffolds: *in vitro* and *in vivo* evaluations

Salwa Suliman[1], Zhe Xing[1], Xujun Wu[2], Ying Xue[1], Torbjorn O. Pedersen[1], Joachim Nickel[3], Yang Sun[4], Anne Doskeland[5], Anne-Finne Wistrand[4], Kamal Mustafa[1]

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P7.6

Evaluation of biocompatibility of newly developed surgical threads made of PGA-co-PLA & PHB

Boguslawa Zywicka[1], Elżbieta Mielicka[2], Anna Pinar[2], Ewa Zaczynska[3], Anna Czarny[3], Agnieszka Walak[2], Izabela Krucinska[4], Danuta Ciechanska[5], Piotr Walak[6], Barbara Uminska-Wasiluk[6], Izabela Oleksiewicz[2]

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P7.7

Effects of high molecular weight hyaluronan for joint capsule in a rat immobilized knee model

Kenji Kanazawa[1], Yoshihiro Hagiwara[1], Masahiro Tsuchiya[2], Yutaka Yabe[1], Kazuaki Sonofuchi[1], Masashi Koide[1], Akira Ando[1], Yoshifumi Saijo[3], Eiji Itoi[1]

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P7.8

Comparison of intraarticular reactions to Multi Walled Carbon Nanotubes(MWCNTs) by injection at once with three divided times.

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P7.9

Evaluation of peripheral nerve regeneration with Diffusion Tensor Imaging (DTI); in vivo rabbit study.

Tetsuro Yamasaki[1], Ryo Oda[1], Hiroyoshi Fujiwara[1], Shigeki Hayashi[1], Tsuyoshi Sukenari[1], Yusuke Hara[1], Shinsuke Morisaki[1], Kazuya Ikoma[1], Kei Yamada[2], Mitsuhiro Kawata[3], Toshikazu Kubo[0]

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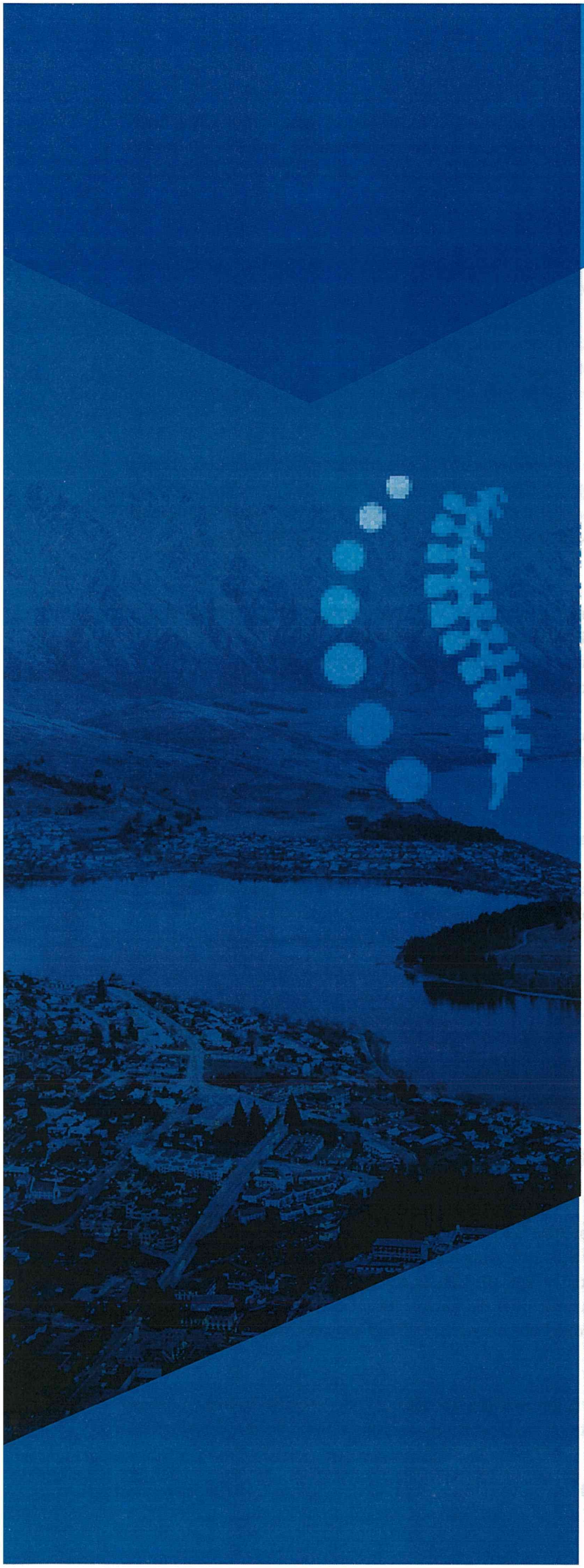

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control group. Studies with primary human osteoblast cultures confirmed the bioactivity of these scaffolds, and the in vivo regeneration of segmental critical size bone defects in a rabbit model demonstrated that this material induces new bone defect bridging, with clear evidence of regeneration of original radial architecture and bone marrow environment.

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Effects for osteosarcoma cells by carbon nanotubes

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Sarcomas such as osteosarcoma are treated with surgery and chemotherapy by anticancer drugs. The anticancer drugs cause various severe side effects, and prospective enough effects may not be obtained. So-called nano-particles smaller than cells have a property to enter cells and they are expected as drug delivery system (DDS). We heretofore reported biocompatibility and safety of the carbon nanotubes (CNT). We report potential as DDS for chemotherapy to osteosarcoma cells with CNTs.

The 143B cells (human osteosarcoma cell line) were seeded at 5.0×10^5 cells/10cm culture plate. After 24 hours, the culture medium was renewed to the medium contained 1 μg/ml or 10 μg/ml multi-walled CNT (MWCNT). Doxorubicin hydrochloride (DOX) (0.1μM, 1.0μM, 5.0μM) was used as positive control. Each group was n=3. After more 24 hours, we observed the cells with light microscope and counted the number of 143B cells of each plate.

In the light microscope images of the 143B cells that we added MWCNTs before 24 hours, the MWCNTs were taken in the cells. In the MWCNT 10 μg/ml group, much MWCNTs were taken in the 143B cells than the MWCNT 1 μg/ml group. The cell number after 24 hours culture was 23.3×10^5 cells/plate in control, 10.3×10^5 cells/plate in DOX 0.1 μM group, 5.6×10^5 cells/plate in 1.0 μM group, 2.8×10^5 cells/plate in 5.0 μM group, 21.3×10^5 cells/plate MWCNT 1 μg/ml group and 16.3×10^5 cells/plate MWCNT 10 μg/ml group.

When the MWCNTs are added to the osteosarcoma cell line; 143B cells, the MWCNTs are taken into the cells and inhibited a cellular proliferation in concentration-dependency. By adhering anticancer drugs to the MWCNTs, we expect to improve invasive efficiency to sarcoma cells of the anticancer drugs, to enhance the chemotherapeutic effect and to reduce the chemotherapeutic side effects.

Disclosure: The authors declare no competing interests.

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Time-elapsed screw insertion into cancellous bone

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"Time-elapsed" or "image-guided failure" assessment of bone is a relatively new technique that uses sequential image acquisition to analyse trabecular bone mechanics under a given loading regime. To date, this procedure has been employed to analyse trabecular mechanics during uniaxial compression [1, 2], screw pull-out [3], and screw push-in tests [4]. Nazarian et al. (2004) validated the use of this method for the assessment of microstructural trabecular mechanics, demonstrating no difference in the macroscopic behaviour of cancellous bone specimens under continuous or step-wise loading conditions [2].

These methods have provided valuable insight into the failure mechanisms of bone under specific loading conditions. Work within our laboratory, however, has sought to better understand the interactions between bone and implant during