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学位の種類	博士（工学）
学位記番号	博甲第7101号
学位授与年月日	平成26年 7月25日
学位授与の要件	学位規則第4条第1項該当
審査研究科	数理物質科学研究科
学位論文題目	Study on Cellular Interaction of Single-Walled Carbon Nanotubes and Their Biomedical Applications （単層カーボンナノチューブの細胞との相互作用及びバイオメディカルへの応用に関する研究）

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論　文　の　要　旨

With the developments of nanoscience and nanotechnology, a variety of nanomaterials with different sizes (1–100 nm), shapes and compositions have been widely used in different fields, including healthcare, electronics, cosmetics, textiles, information technology and environmental protection. Because of their small size, large specific surface area and high surface atomic slot insufficiency, nanomaterials exhibit fascinating and novel properties that are often vastly different from their bulk counterparts, such as low weight, high plasticity and high electrical/thermal conductivity as well as unique optical properties. The unique properties of nanomaterials offer excellent platforms for the diagnosis and therapy of diseases. Meanwhile, since cells in biological systems create and directly interact with nanostructured extracellular matrices (ECM), nanomaterials with excellent biomimetic features and physiochemical properties show great potential in the fabrication of novel biomaterials and scaffolds for tissue engineering. Among various nanomaterials, carbon nanotubes (CNTs), especially single-walled carbon nanotubes (SWCNTs) have attracted intense interest and shown promising applications in the realm of targeted drug/gene delivery, diagnostics, cancer research and tissue engineering.

For biomedical usage, the interaction of nanomaterials with biological systems, especially with cells is the focus of current investigations. The success of nanomaterials-based applications in disease diagnosis and therapy largely depends on whether the designed nanomaterials can be easily internalized by cells. However, the internalized nanomaterials may produce some negative effects on cells. For example, when nanomaterials enter the biological system and accumulate within cells, they may disrupt the organelle integrity, alter gene expression and lead to other intracellular changes. Therefore, one of the crucial issues regarding nanomaterials-based applications that have to be addressed is to understand how nanomaterials interact with cells and to uncover their potential risks. Meanwhile, feasible techniques are needed to detect the subtle changes of cells when they are exposed to nanomaterials.

In this study, SWCNTs were used and the interaction of SWCNTs with cells, especially the cellular uptake and the associated cellular effects were investigated. Atomic force microscopy (AFM) was employed to investigate the changes of mechanical properties of cells when they were exposed to SWCNTs. SWCNTs were further explored for stem cell research and incorporated in scaffolds for tissue engineering application.

1. Interaction of SWCNTs with cells in 2D cell culture system

The purpose of the first part of study is to investigate the interaction of nanomaterials with cells and cellular effects of nanomaterials in 2-dimentional (2D) cell culture system. SWCNTs were used and functionalized with collagen (Col-SWCNTs) to improve their dispersibility in aqueous solution. The Col-SWCNTs retained the inherent properties of SWCNTs and the suspension solution was stable for months. The cellular effects, uptake and intracellular distribution of Col-SWCNTs were investigated by using them for culture of bovine articular chondrocytes (BACs). High amount of Col-SWCNTs were internalized by cells without obvious negative cellular effects. The internalized Col-SWCNTs were distributed in the perinuclear region and retained in the cells for more than one week. It was found that the adsorption of SWCNTs by extracellular matrix (ECM) played an important role in cellular uptake of SWCNTs. The high stability, easy cellular uptake and long retention time in cells of the Col-SWCNTs will facilitate the biomedical applications of SWCNTs.

2. Investigation of interaction of nanomaterials with cells using atomic force microscopy

Besides the traditional investigation methods, feasible techniques that can detect the subtle changes of cells when they are exposed to nanomaterials are also highly needed. AFM has been developed as a powerful tool in the imaging of cells and probing their mechanical properties under physiological conditions. In this part, AFM was employed to investigate the effects of nanomaterials on cells. Two different types of nanomaterials (SWCNTs and Fe-FeO core-shell magnetic nanoparticles) and three types of cells (BACs, human bone marrow-derived mesenchymal stem cells (hMSCs) and HeLa cells) were used. WST-1 assay and live/dead staining showed that the cells had almost the same levels of viability. However, AFM measurement showed that the cells changed their Young's modulus when different types of nanomaterials at different concentrations were applied in the cell culture medium for different time. The results indicated that the effects of nanomaterials on the mechanical properties of cells were dependent on nanoparticles size, concentration and cell type as well as exposure time. More importantly, AFM was demonstrated to be useful to identify the subtle changes of the mechanical properties of cells when they were exposed to nanomaterials even for very short time.

3. SWCNTs-based stem cell labeling and imaging

Stem cells have shown great potential in biological applications because of their capacity to self-renew and differentiate into multiple cell lineages and stem cell implantation has been considered as a promising therapeutic strategy for various diseases and defects. A critical aspect of stem cell-based therapies is to distinguish the implanted cells from the host cells and to monitor them in terms of their viability, migration, distribution and the relative contributions from the delivered cells versus host cells. With this respect, a

reliable and cytocompatible *ex vivo* labeling method for stem cells is critically needed. On the other hand, as a kind of quasi one-dimensional (1-D) nanomaterials, SWCNTs exhibit various unique optical properties which are helpful in biomedical labeling and imaging. SWCNTs have sharp electronic density of states at the van Hove singularities, resulting in strong and distinctive resonance Raman scattering that includes the radial breathing mode (RBM) and tangential mode (G-band). These peaks are sharp and strong and can be easily distinguished from fluorescence backgrounds, which are suitable for optical imaging. Another important advantage of SWCNTs as fluorophores is the lack of photobleaching without any noticeable decay or loss of photoluminescence intensity even under extended laser excitation. All of these features make SWCNTs suitable for stem cell labeling and imaging.

In this part, Col-SWCNTs were used as imaging probes for labeling of hMSCs and the inherent Raman scattering of SWCNTs was used to image the SWCNT-labeled cells. The results showed that Col-SWCNTs exhibited efficient cellular internalization by hMSCs without affecting their proliferation and differentiation. The prolonged dwell time of Col-SWCNTs in cells ensured the long-term labeling for up to 2 weeks. This part of work revealed the potential of Col-SWCNTs as a probe for long-term stem cell labeling and imaging.

4. Interaction of SWCNTs with cells in 3D cell culture system

It's well known that 2D cell culture conditions are different from the microenvironment surrounding cells *in vivo*. As a result, the response of cells to nanomaterials during 2D cell culture, including cellular uptake, can not completely reflect the virtual behavior in the native biological system. One potential strategy to overcome the problem is to culture cells with a 3-dimensional (3D) culture system which can mimic the natural ECM. Meanwhile, a scaffold with appropriate properties, such as good biocompatibility, high mechanical property and feasible inner structure is highly demanded for tissue engineering by furnishing a biomimic microenvironment to control cell functions and to guide new tissue formation. One of the strategies to develop such functional scaffolds is to incorporate nanomaterials into the scaffolds because of the structural similarity of nanomaterials with *in vivo* microenvironments.

In this part, SWCNTs/collagen composite hydrogels and porous sponges were prepared for 3D cell culture. The interaction of SWCNTs with cells was investigated by culturing BACs in the composite hydrogels and sponges. The results showed that SWCNTs could be internalized by cells when SWCNTs were incorporated in these 3D scaffolds. And the incorporation of SWCNTs in the porous sponges promoted cell proliferation and sGAG production through offering nanoscaled topography. The results suggested that composite scaffolds incorporated with nanomaterials may be useful for tissue engineering applications.

In summary, SWCNTs were functionalized with collagen (Col-SWCNTs) and the interaction of SWCNTs with cells was investigated in 2D and 3D cell culture systems. The Col-SWCNTs showed high stability, easy cellular uptake and good cytocompatibility, which will facilitate the SWCNTs-based biomedical applications, such as drug/gene delivery, biological imaging and cancer therapy. Meanwhile,

AFM was employed to investigate the effects of nanomaterials on cells. The results showed that AFM was able to identify the subtle changes of the mechanical properties of cells when they were exposed to nanomaterials and could be a useful tool to investigate cellular effects of nanomaterials. Furthermore, the Col-SWCNTs were used as an imaging probe for labeling of hMSCs. The results showed that the Col-SWCNTs exhibites efficient cellular internalization by hMSCs without affecting their proliferation and differentiation and Col-SWCNTs should be a good candidate for long-term stem cell labeling. Finally, SWCNTs were incorporated in collagen hydrogels and porous sponges to construct 3D cell culture system to investigate the interaction between SWCNTs and cells. The results of cells cultured in SWCNTs/collagen composite scaffolds should reflect the responses of cells to the nanomaterials in native biological system more realistically and the composite scaffolds will facilitate the nanomaterials-based applications in tissue engineering.

審　　査　　の　　要　　旨

〔批評〕

本論文では、カーボンナノチューブをバイオメディカル分野に応用するために、その生体機能化に着目して研究を行った。まず、単層カーボンナノチューブを細胞親和性にすぐれたタイプ Ⅰ コラーゲンで表面修飾した。コラーゲンで修飾した単層カーボンナノチューブは水溶液に数か月の期間安定して分散することができた。本コラーゲン修飾カーボンナノチューブを添加してウシ関節由来の軟骨細胞を培養したところ、修飾カーボンナノチューブが細胞にきわめて高い効率で取り込まれることを見出した。本修飾カーボンナノチューブの細胞への取り込みには、その細胞自身によって産生される細胞外マトリックスが関わっていることを明らかにした。また、原子間力顕微鏡の手法を用い、カーボンナノチューブや酸化鉄ナノ粒子といったナノマテリアルと細胞との相互作用を調べた。カーボンナノチューブと酸化鉄ナノ粒子を取り込ませたウシ軟骨細胞では弾性率が増加することを示した。ウシ軟骨細胞の他、ヒト骨髄由来の間葉系幹細胞、HeLa 細胞を用いて同様の実験を行った結果から、細胞種によって弾性率の増加がわずかに異なることが分かった。上記のナノマテリアルの濃度及び培養時間も細胞の弾性率変化に影響することを明らかにした。これらの知見から、原子間力顕微鏡がナノマテリアルと細胞との相互作用を調べるための有用な方法であることが示唆された。さらに、コラーゲン修飾カーボンナノチューブの細胞への高い取り込みを利用して、幹細胞の標識への応用について検討した。コラーゲン修飾カーボンナノチューブをヒト骨髄由来の間葉系幹細胞に添加して培養を行った。カーボンナノチューブがラマン吸収をもつことを利用し、細胞をラマン顕微鏡で観察した。その結果、取り込まれたカーボンナノチューブは間葉系幹細胞の増殖と分化には影響せず、長期間にわたって幹細胞内に局在化し、幹細胞を標識することが可能であった。最後に、コラーゲン修飾カーボンナノチューブをコラーゲンヒドロゲルとコラーゲンスポンジにそれぞれ導入し、生体内に近い条件として三次元培養を行い、カーボンナノチューブとウシ関節軟骨細胞との相互作用を調べた。三次元培養においてもカーボンナノチューブは細胞によって取り込まれることが明らかになった。カーボンナノチューブと複合化したコラーゲンスポンジでは、軟骨細胞の接着、増殖が促進されるとともに、硫酸化グリコサミノグリカンの産生量も増加した。本研究で得られた一連の成果は、ナノマテリアルをバイオメディカル分野に展開する上で重要な学術的知見を与えるものと考えられる。よって、本論

文は博士（工学）の学位論文として十分な学術的な価値をもつものと認める。

〔最終試験結果〕

平成26年6月23日、数理物質科学研究科学位論文審査委員会において審査委員の全員出席のもと、著者に論文について説明を求め、関連事項につき質疑応答を行った。その結果、審査委員全員によって、合格と判定された。

〔結論〕

上記の論文審査ならびに最終試験の結果に基づき、著者は博士（工学）の学位を受けるに十分な資格を有するものと認める。