Construction of biomolecule-immobilized TiO$_2$ nanoparticle for applying to new cell injuring method with ultrasound irradiation

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Recently, we discovered the alternative titanium dioxide (TiO$_2$) activation method by ultrasound irradiation instead of ultraviolet irradiation, and also observed this activation could be produced the OH radical by combination between methylene blue, which was a typical substrate for photocatalytic reaction, and various radical scavengers. Although the irradiation of ultrasound through water media had been known to produce various ROS, including OH radical and superoxide anion oxygen radical (O$_2^\cdot$), we assumed that these ROS production including OH radical were enhanced by ultrasound irradiation with TiO$_2$ (noted as a US/TiO$_2$ method). Furthermore, the production of ROS by US/TiO$_2$ had been applied to chemical degradation and two kinds of microorganism’s disinfection by pellet type of TiO$_2$ such like a ceramic. This US/TiO$_2$ method has an advantage compared with the typical photo excitation method, ultraviolet irradiation, because the generated sound wave by ultrasound has the transduced potential through the matter that has no transparent, and resulting could be activated the TiO$_2$ located at the inside of mater, such like a body. The photodynamic therapy (PDT) was well known as one of new cancer therapy approaches. Typical PDT was carried out by combination of photosynthesizer, such like a porphyrin IX, and activator such like a laser. The mechanism of PDT therapy was based on the cell injuring or killing by the produced ROS. However, the application limitation of PDT therapy was closely restricted to the laser irradiation area, and the PDT was only just applied several clinical cases such like a skin cancer.

Since the ROS generated on the surface of TiO$_2$ have very short half-lives, a large portion of the ROS disappear during diffusion in aqueous media without participating in any injuring or killing. Therefore, the specifically delivery method of TiO$_2$ nanoparticle to interested tissue was required for the effective cell injuring by US/TiO$_2$. However, TiO$_2$ had no cell reorganization potential. Moreover, the OH residue on the surface of TiO$_2$ nanoparticle was easily associated each other at neutral pH, and resulting these comforted aggregated particle, which was not adequate for clinical application. Kanehira et al. constructed the TiO$_2$ nanoparticle, which was permitted us to suspend at neutral pH by surface modification by polyacrylic acid (PAA) with high temperature. Since the dissociation constant of the carboxylic acid group of PAA is around pKa 4.0, PAA is a suitable candidate for chemical modification at the surface of TiO$_2$ particle. In addition, the carboxylic acid group of PAA allows for immobilization of proteins via covalent bonding with amino groups. Together with these previous ROS utilization findings to cancer therapy and sanitary cleaning and the TiO$_2$ nanoparticle modification method, we will be undertaken the applying of ROS by US/TiO$_2$ to the mammalian cell killing or injuring. To achieve this purpose, we firstly investigated the construction of protein immobilized TiO$_2$ nanoparticle for targeting to the specific tissue, and the possibility of OH radical
production by this protein modified TiO$_2$ nanoparticle in this study.

The particle size of constructed PAA-TiO$_2$ nanoparticle was approximately 100 nm, and the diameter was increased with the modification of protein. The increment diameter was 20 nm, and it was assumed that the increase might be corresponded to two proteins diameter and the immobilized protein covered to TiO$_2$ nanoparticle as a single layer. Since the diameter of GST-GFP-preS1/S2 was estimated to be 7 nm by Flory’s theory, the increased diameter of nanoparticle was well agreed with this theoretical estimation. The number of protein molecule immobilized onto each of TiO$_2$ nanoparticle was estimated to be 420. Comparing with the theoretical number of protein covered (approximately 440, as a globule protein 10 nm) onto particle (100 nm) as a single layer, the immobilized protein number in this study was well agreed, and this calculation also supported that the protein covered all of almost TiO$_2$ nanoparticle. Therefore, the GST-GFP-preS1/S2 protein displayed on the surface of TiO$_2$ nanoparticle could recognize the anti-preS1 antibody, and possessed a localizing function to HepG2. Moreover, the protein modified TiO$_2$ nanoparticle kept the OH radical generation potential nevertheless the surface was covered by protein. Together these results, we concluded that the constructed protein modified TiO$_2$ nanoparticle had dual function: the targeting to the specific cell, and the OH radical generation for cell injuring. Comparing the localization of GST-GFP-preS1/S2 protein in HepG2, although the free protein translocated well to the cytosol, the GST-GFP-preS1/S2 immobilized TiO$_2$ nanoparticle just localized at around the cell membrane fraction. Nevertheless the fusion between Hepatitis B virus and liver cell was completely carried out within 6 h, these nanoparticle could not perform completely fusion to HepG2 cell in this study. Therefore, we assumed that the constructed nanoparticle may be inhibited the uptake itself by endocytosis.

By applying the ultrasound irradiation at 0.4 W/cm$^2$, 1 MHz to the TiO$_2$ nanoparticle incorporated HepG2, the cell damage was observed, and the cell damage was enhanced by addition of TiO$_2$ nanoparticle. Amount of generated OH radical in the presence of TiO$_2$ nanoparticle at 0.4 W/cm$^2$ was equivalent to that in the TiO$_2$ nanoparticle absence condition at 0.6 W/cm$^2$. Therefore, we assumed that the cell damage could be induced by these OH radical concentrations regardless of experimental conditions. To improve the cell damage efficiency moreover at lower ultrasound irradiation, there are several improving points not only the immobilization efficiency of GST-GFP-preS1/S2 protein to the TiO$_2$ nanoparticle, but also the ultrasound irradiation method such like a directly or indirectly. In additionally, we also should be proceeded the investigation of injuring mechanism of cell by TiO$_2$ nanoparticle because there was no any scientific evidences.

This is the first study demonstrating the specific cell injuring by TiO$_2$ nanoparticle, which has dual functions: specific cell recognition and OH radical generation. By modification with various targeting protein such like an antibody or epidermal growth factor (EGF), this proposed methodology will be permitted us to ping point therapy of various cancer cell (tissue) in the near future.

**Keyword:** Titanium dioxide (TiO$_2$), HepG2, Immunostaining, Cell membrane damage, Hydroxyl radical