Letter to the Editor

Effect of Sterilization Methods on Cell Binding Activity of Surface-immobilized Fibronectin*

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The effect of sterilization methods on biological activity of fibronectin on the surface of biomaterials was elaborated in the present study. Sterile protein- modified biomaterials were fabricated by microfilter filtration and UV irradiation, respectively. UV irradiation altered the conformation of surface- adsorbed fibronectin and further affected the attachment, morphology and biological function of endothelial cells. However, microfilter filtration did not to change the normal conformation of fibronectin, or the proliferation and biological function of endothelial cells, indicating that microfilter filtration sterilization is the most suitable method for protein-substrate.

The surface of implants plays an important role in determining its biocompatibility due to its direct contact with host tissues. Therefore, special attention should be paid to the surface modification of biomaterials. The effective strategy for improving their biocompatibility is to immobilize their biomolecules on the surface of biomaterials[1]. However, little is known about the effect of sterilization methods on the surface of immobilized biomolecules.

Different sterilization methods are now available for various implants. The advantages of steam sterilization are its simple, effective and rapid procedure without toxic residues. However, it is mainly used for metallic implants. Polymers may undergo melting and softening during autoclaving[2]. Ethylene oxide (EtO) is a polymer implant, which nevertheless, is toxic with a suspected carcinogen residue[3]. Gamma irradiation is a useful sterilization procedure for film biomaterials. However, both EtO and gamma irradiation can damage the structure and morphology of tissue engineering graft[2]. UV irradiation represents a simple, cheap and effective procedure. However, it has been reported that UV irradiation degrades polymers or changes the surface physical and chemical properties of inorganic biomaterials[4]. Ultrafiltration is suitable for sterilizing biomolecules without radiating and deteriorating their molecular structure. However, it cannot be used for sterilizing the surface of immobilized biomolecules because it is time consuming and requires a sterile operation line. Generally, a specific sterilization method is selected based on economic considerations and implant material properties. Most sterilization methods focus on their sterilization effect. However, their effect on the biological activity of immobilized biomolecules on the surfaces of biomaterials remains largely unknown.

The effect of UV and filtration sterilization on the biological activity of immobilized fibronectin (Fn) on the surfaces of biomaterials was studied in the present study. Fn, as an important extracellular matrix (ECM) protein, can enhance the attachment and proliferation of endothelial cells (EC) without altering the graft patency *in vivo*, while endothelialization is considered as an ideal procedure for cardiovascular implants. Therefore, it is of great interest and necessity to study the effect of sterilization methods on Fn activity that is immobilized on the surfaces of biomaterials. The effect of sterilization methods on wettability, conformational change, quantity and activity of RGD peptide, and on adhesion, proliferation and biological function of EC were studied in order to validate the change in related activity of. Fn, which was expected to shed more light on UV irradiation as a sterilization procedure for biomolecules of modified biomaterials.

Titanium (Ti) substrates (10×10 mm, Baoji, China) were cleaned and dried before use. Figure 1 shows the two fabrication processes of different samples. The Ti substrates were sterilized in autoclave containing 100 μL Fn (Sigma-Aldrich) solution, filtered with a 0.22 μm microfilter (Carrigtwohill, Ireland), added onto Ti, and incubated at 37 °C for

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1 h. The redundant Fn solution was removed from the surface with a micropipette. The samples denoted Ft-Fn. Alternatively, the Ti substrates were placed into a culture plate. Fn at the same concentration was directly added onto Ti, and incubated at 37 °C for 1 h. The samples were irradiated under a mercury-vapor UV lamp (254 nm, 15 w, 60 Hz, Foshan, China) at a 30 cm distance for 30 min. The samples thus obtained denoted UV-Fn. Pure Ti was used as a blank sample (BLK). All the samples were used immediately after sterilization.

Static water contact angles of the prepared samples were measured using a contact angle apparatus (GBX, France). The Fourier transform infrared spectra (FTIR) of bare Ti, Fn-adsorbed Ti (without sterilization), and 30 min UV- sterilized Fn were detected with a FTIR spectrometer (Bruker, Germany). The quantity and RGD peptides exposed to Fn in different samples were assayed by immunochemistry as previously described[5]. The experiments were done in quadruple.

The 3rd generation of EC (5×10^4 cells/mL) derived from human umbilical vein was used to assess their proliferation behavior. The proliferation, cytoskeleton formation and biological function of EC on days 1 and 5 after incubation were investigated using CCK-8, SABC-FITC, and MCP-1 kits (Feiao, China), respectively. The number of EC was calculated and the concentration of MCP-1 was measured according to the calibration curve. The skeleton of EC was photographed with an inverted fluorescence microscope (Olympus, Japan).

The data were analyzed by one-way ANOVA analysis using software SPSS 11.5 (Chicago, Illinois). $P<0.05$ was considered statistically significant.

The water contact angles are shown in Figure 2A. The bare Ti substrate without UV irradiation and Fn

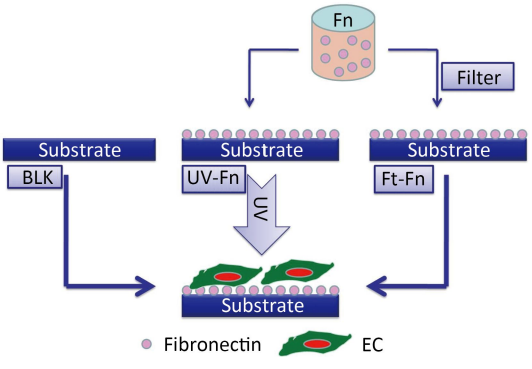

Figure 1. Sample preparation, UV irradiation time, Ti and distance between Ti and UV lamp.

adsorption were more hydrophobic with a contact angle of about 87.5°. The water contact angle of Fn-adsorbed substrate decreased to about 70°, with a relatively higher surface hydrophilicity, whereas it increased to about 75° after UV irradiation of Fn-adsorbed Ti, showing a more hydrophobic surface after UV irradiation. Adsorption of Fn introduced a large number of hydrophilic groups, such as -COOH, thus improving the surface hydrophilicity. Otherwise, Fn is amphipathic with different hydrophobic and hydrophilic domains on its long chain. UV irradiation may change the conformation of Fn[6] and more hydrophobic domains, thus resulting in a higher contact angle. FTIR was used to verify our speculation. As shown in Figure 2B, new weak peaks appeared at 1 640-1 750 cm^{-1} (amide groups) on Ti-Fn and UV-Fn samples, indicating the derivation of Fn on Ti surface. In addition, new peaks appeared from 500-750 cm^{-1} on the UV-Fn sample on days 0, 1, and 3 after culture, whereas none of these peaks was observed on Ti-Fn and UV-Fn sample on day 5 after culture, indicating that UV irradiation could change the conformation of protein, which might possibly recover after a certain period of time. We believe that UV irradiation can change the conformation of surface-immobilized proteins and further induce the variation of surface properties, which may influence the attachment, spreading, and proliferation of EC.

Figure 2C depicts the Fn quantity and RGD peptides exposed to different samples. The volume of adsorbed Fn, UV-Fn, and Ft-Fn was significantly larger in Ctrl sample (directly adsorbed Fn on surface without sterilization) than in BLK sample ($P<0.05$) while no significant difference was observed in samples of Ctrl Fn, UV-Fn, and Ft-Fn ($P>0.05$), indicating that neither filter sterilization nor UV irradiation affected the quantity of surface-adsorbed Fn. To reveal the possible conformational change of Fn caused by UV irradiation, the exposure of RGD peptides was also measured as previously described[7]. More RGD peptides were exposed to Ft-Fn than to UV-Fn, indicating that UV irradiation decreased the cell- binding activity of Fn. Interestingly, both UV-Fn and Ft-Fn samples showed a significantly greater RGD exposure than the Ctrl sample ($P>0.05$), which might be explained by the fact that Fn was folded under natural conditions while stretched or conformational changes after filter and UV sterilization. UV irradiation altered the conformation of surface-adsorbed Fn due to the change in wettability. Therefore, UV irradiation did affect the biological activity of Fn rather than its

quantity, which was consistent with water contact angle measurement.

Figure 3A shows the amount and proliferation of EC on the surfaces of Ti substrate, UV-Fn and Ft-Fn. More EC were attached to the surface of UV-Fn and Ft-Fn than to that of bare Ti substrate on days 1 and 5 after culture ($P<0.05$). Less EC were attached to UV-sterilized and Fn-adsorbed surfaces than to the filter-sterilized and Fn-adsorbed surfaces after cultured on day 1 after culture ($P<0.05$). However, no significant difference was found in the attachment of EC to Ft-Fn and UV-Fn samples on day 5 after culture ($P>0.05$). Interestingly, the volume of EC was significantly greater in bare Ti substrate, Ft-Fn and UV-Fn on day 1 than on day 5 after culture. UV sterilization of Fn decreased the attachment of EC, which was consistent with the RGD exposure, revealing that UV irradiation changed the conformation of Fn and further inhibited the attachment and proliferation of EC, especially on day 1 after culture. Why EC proliferated more rapidly on UV-Fn sample on day 5 after culture still remains unclear. The possible reason may be that the conformation of protein was recovered after immersion in M199 as shown by FTIR, which weakened the influence of UV irradiation. In addition, the interactive micro-environment between surface-immobilized protein and cell culture medium is complicated, therefore further study is needed to show its underlying mechanism.

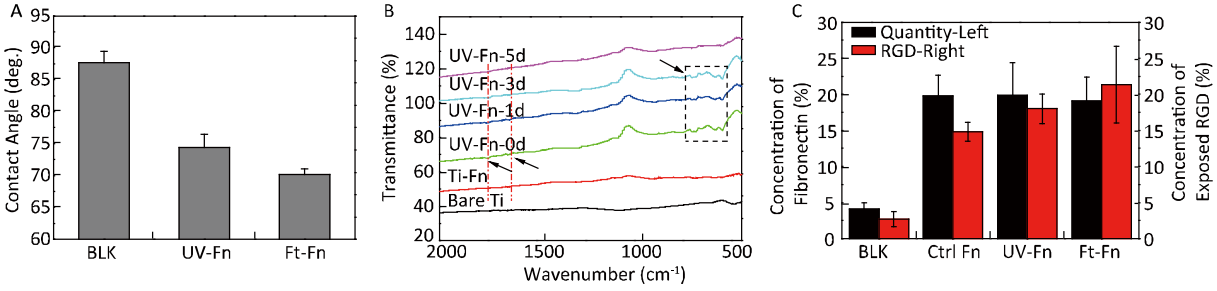

Figure 2. Characterization of water contact angle as a function of sterilization (A), FTIR spectra of bare Ti, Ti-adsorbed Fn, and UV- sterilized Fn (B), quantity and RGD peptides exposed to fibronectin on bare Ti), UV-Fn and Ft-Fn (C).

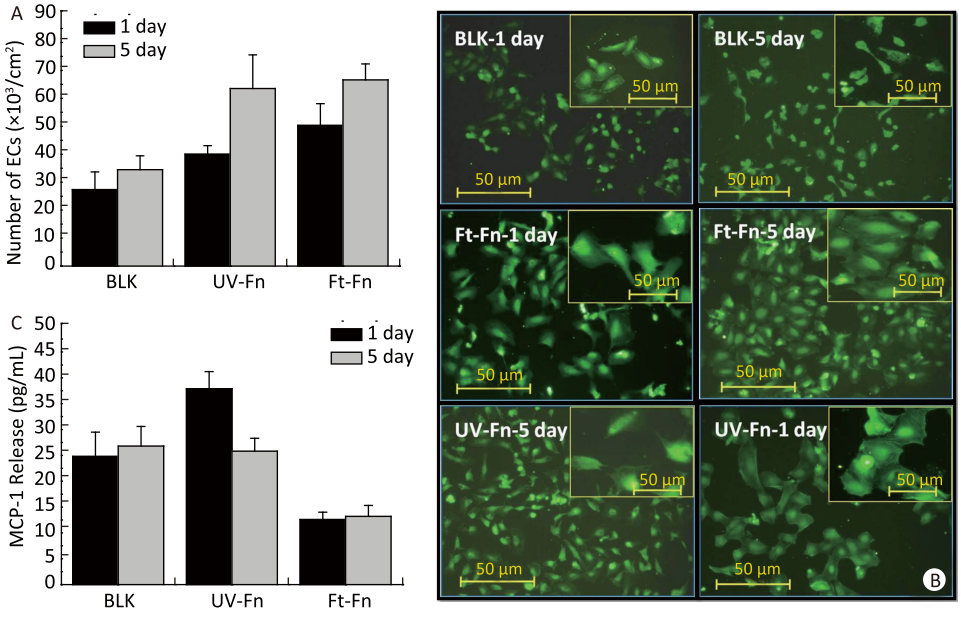

Figure 3. Effect of sterilization methods on proliferation (A), skeleton distribution (B), and MCP-1 secretion (C) of EC on bare Ti, UV-Fn, and Ft-Fn.

Figure 3B shows the cytoskeleton of EC on days 1 and 5 after incubation. EC exhibited an elliptic or polygonal morphology on Fn-adsorbed surface and a rounded or contractive morphology on bare Ti substrate surface on day 1 after culture, indicating a better support of Fn for attachment and spreading of EC. Few EC proliferated on bare Ti surface on day 5 after culture. Furthermore, most EC exhibited a rounded or contractive morphology, especially on day 1 after culture. However, a large number of EC still displayed a well spread morphology in samples of UV-Fn and Ft-Fn on day 5 after culture. Little contract of EC on UV-Fn was observed with no contract on Ft-Fn. EC on Ft-Fn surface exhibited a spindle and cobblestone morphology, which is similar to the normal morphology *in vivo*. Notably, the morphology of EC is closely related with their function. The normal morphology of EC on the vein wall is spindle or oval, thus presenting a better biological function. The round or congregated EC inhibited their proliferation and biological function, and even induced apoptosis[8]. The spreading and morphology of EC are related with their surface wettability[9] and physiochemical properties[10], which are the important modulators of cellular function. In the present study, both surface wetting and physiochemistry were changed after Fn adsorption while UV irradiation changed the conformation of protein and increased the surface hydrophobicity, as a result more adhesion and proliferation of EC with a better morphology were detected on Fn-adsorbed Ti surface without UV irradiation. It was anticipated that UV irradiation sterilization changed the conformation of surface-adsorbed Fn and affected the attachment and proliferation of EC, especially their morphology and biological functions.

The expression of MCP-1 was detected in this study, because it was considered to be a prominent participant in the recruitment of monocytes and leukocytes to endothelium and was correlated with atherosclerosis[11]. The MCP-1 secretion of EC cultured for 1 and 5 d (Figure 3C) showed that both bare Ti and UV-Fn surfaces displayed a significantly higher MCP-1 concentration than Ft-Fn surface (P<0.05), indicating that simulated EC released inflammation-related factors. Moreover, EC cultured in UV-Fn sample showed the release of MCP-1 on days 1 and 5 after culture, suggesting that UV irradiation changed the conformation of surface-immobilized protein and the normal function of EC. It is thus reasonable to believe that the surface chemistry of biomolecules-immobilized biomaterials can be changed by sterilization methods, which may further impact the function of EC, especially at the early stage.

In the present study, UV irradiation caused conformational change of fibronectin and reduced cell-binding sites, which further affected the morphology and biological function of EC, indicating that UV irradiation could sterilize the surface-immobilized biomaterials. Microfilter filtration could maintain the normal conformation of protein without influencing proliferation and biological function of EC. It is therefore of paramount importance for the selection of suitable methods to sterilize various proteins-modified biomaterials.

In conclusion, microfilter filtration sterilization is a better procedure than UV irradiation for sterilizing proteins-modified biomaterials.

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