Application of flexible flask for static culture of adherent cells

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Abstract: Conventional T-flasks with different sizes and layers are commonly used devices for static cell culture at laboratory scale. Due to their non-deformable structure and requirement of strict manufacture art, the rigid T-flask costs highly in production and transportation and, of course, produces more waste. In this study, we described a pentahedron shaped flexible flask made of a medical application plastic film and reported its application in anchorage-dependent cell culture. The results showed that the flexible flasks achieved compatible efficiencies with T-flasks in the yield, viability and homogenous distribution of Vero cells and 293 cells. We concluded that the atactic polypropylene and styrene-ethylene-butene-styrene-copolymer plastic film was a good biocompatible material with cells and the novel flexible flask is a cost-effective alternative culture vessel for static culture of adherent cells at laboratory scale and possesses several special advantages such as simple to manufacture, foldable to save shipping and storage room and low in waste generation.
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1. Introduction

Conventional single layer and multi-layer T-flasks with different sizes and number of layers are commonly used culture vessels made of rigid plastic, such as polystyrene, or glass for static cell culture at laboratory scale (Ammerman et al., 2008). When large amounts of cells are needed by using T-flasks as culture vessels for some purposes, such as producing sufficient recombinant proteins, antibodies, virus or seed culture, alternative solution is to use enlarged single layer flasks, or multi-layer T-flasks (Abraham et al., 2011) or multiple T-flasks to satisfy such requirements. However, to make the oversized culture surface flat is challenging by using such rigid material from a manufacturing standpoint, thus commercial available single layer tissue culture flasks typically ranges from 25 cm^2 to 225 cm^2 in area (Bleckwenn and Shiloach. 2004). As we know, anchored cell growth on the bottom surface of single layer flasks or layered plate surface of the multi-layer T-flasks just requires a small volume of medium, for example, a T-175 flask has a bottom area of 175 cm^2, the recommended maximum medium height is about 3 mm (about 50ml of volume), however, to ensure enough room for adequate aeration, a typical T-175 flask is made with a height of 3.7 cm (total volume about 650ml), that means the medium just occupies 8% of total volume of the flask, whereas the remaining 92% is used for accommodation of gas. Therefore the non-deformable rigid hollow flasks lead to an increased shipping/storage room, corresponding cost and waste generation. In addition, employing multiple T-flasks for scale-up culture would lead to increases in handling frequency, labor, and contamination risk. In order to overcome the drawbacks of rigid T-flasks mentioned above, an ideal solution is to develop flexible flasks. In previous study, we have developed a flexible flask with a medical application plastic film for suspension culture of *Escherichia Coli* (Yang T et al., 2013). In this study, we tested if the plastic film used for manufacturing the bioreactor could be a good material for adherent cells anchorage growth and this kind of three-dimensional flexible flask could be an alternative culture vessel for anchorage-dependent cell growth.

2. Material and methods

2.1. Design and manufacture of the flexible bioreactor

The plastic film for preparing the flexible flask was purchased from Double-Dove Group Co. Ltd, Shanghai, China, which was approved by China's State Food and Drug Administration as Class I medical packaging film with a thickness of 200μm, consisting of atactic polypropylene (PP) and styrene-ethylene-butene-styrene-copolymer (SEBS) (ratio 9:1) in the inner layer (PP/SEBS layer), SEBS in the middle layer and homo-polymer PP in the outer layer, respectively. As shown in Figure 1, the flask

was manufactured by folding the film following the dotted lines and fusing the edges of the film with a thermo-compressor (4V210-08, Meizhidian Electronic Equipment Factory, Wuxi, China). On the upper of one quadrangle side, a hole with 3.5 cm in diameter was punched and a rigid pipe made of modified PP, same as the neck of the control T-flasks in length (3cm) and bore diameter (3.5cm) was sealed onto the surrounding film of the hole through its proximal sealing ring and used as the port for bio-materials/bio-products transfer and aeration. A vent cap (NEST Biotech Co., Ltd., Wuxi, China) was used to screw on the mouth (the opening) of the flask, which was the same as the cap of T-flask (175 cm^2, 3.5cm bore diameter, Nest Biotechnology Co., Ltd., Wuxi, China).

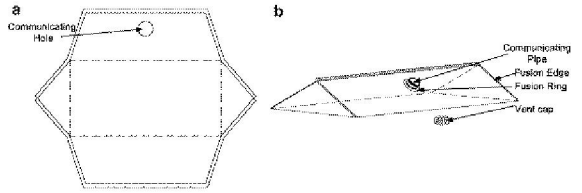

Figure 1. Schematic diagram showing the preparation of the flexible flask
a. A single sheet of medical application plastic film cut in the indicated shape for the manufacture of the flexible flask. **b**. A perspective view of a flexible flask after folding and fusing.

In order to make corresponding ratio of bottom surface area to volume closer to that of the control T-175 flask, the flexible flask was designed and prepared with a length of 18cm, a width of 10cm and a total volume of 650 ml to match a single standard T-175 flask and another one with a length of 33cm, a width of 16cm and a total volume of 1950 ml to match the scale of three T-175 flasks, respectively (Figure 2a). Once the bottom surface area and the total volume was determined, the angles between the quadrangle side walls and the bottom were kept as less as possible, usually less than 45 degrees to facilitate the observation under a microscope. The flexible flasks were sterilized with γ-irradiation (Cobalt 60) at a dose of 20 kGy (the Irradiation Engineering Research Center, Henan Academy of Science Institute of Isotope, Zhengzhou, China). The T-flasks were pre-sterilized by gamma radiation and adopted tissue culture treatment (TC-treatment).

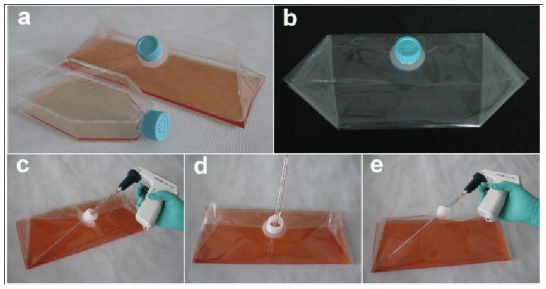

Figure 2. Scale up of the flexible flask
a. Scale up of the flexible flask. **b.** The folded flexible flask. The crease line on its bottom surface is caused during the production process, thus they were used as the folding line. The flexible flask is foldable to facilitate its transportation and storage. It could be expanded easily by stretching the opposite corners. **c-e** Sites of pipette at the bottom of the flask Since the flask was flexible, pipette could reach to every spot of its bottom by changing the direction of the pipe.

2.2. Cell culture

African Green monkey kidney cells (Vero) and 293A cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences and cultured with Dulbecco's Modified Eagle's Medium plus 10% (v/v) fetal bovine serum, 50 IU penicillin ml^{-1}, 50μg streptomycin ml^{-1}, 1% (v/v) 0.2 M L-glutamine (all the products from GIBCO, Paisley, Scotland, UK) and 1% non-essential amino acids 100× (Sigma) at pH 7.2. Vero cells were seeded at a density of approximately 1.0×10^5 cells ml^{-1} with a working medium volume of 50ml for each of the flexible flasks and corresponding sized T-175 flasks and 150ml into each of the enlarged flexible flasks with a scale equivalent to three T-175 flasks, respectively. The medium was used at a ratio of 0.3 ml cm^2. All the cells were cultured in an incubator (MCO175, SANYO Electric biomedical Co., Ltd., Japan) at 37 ℃ in a humidified atmosphere containing 5% CO_2.

2.3. Evaluation of Cell attachment effects

After inoculation, the cell adhesion state was evaluated by direct observation under an inverted phase-contrast microscope (BDS200, Chongqing Optec Instrument Co., Ltd) and a following quantitative calculation by a hemocytometer at 2, 4, 6 and 24 hours, respectively. Firstly, the medium in the culture vessels was carefully removed and the cells were gently rinsed twice with phosphate buffered saline (PBS) to remove unattached ones; Then nine view fields (100×magnification) for each culture vessel were randomly observed and photographed (PowerShot A650 IS, Canon); Finally, the attached

cells were trypsinized for nuclei counting to determine cell attachment rate, a ratio of attached cells to total inoculated cells. For nuclei counting of attached cells (Pisania et al., 2010), the trypsinized cells were centrifuged at ~1,000×g for 15 minutes and the supernatant was discarded. Then the cell pellets were resuspended in a staining solution equal to 50% the original volume, comprising of 0.1% (w/v) crystal violet (Sigma) and 0.1% v/v Triton X-100 (Sigma) in 0.1 M citric acid (Sigma) and incubated for 2 hours. The numbers of nuclei were counted in a hemocytometer.

2.4. Cell yield and viability

When the cells reached to near confluence at 48 hours after seeding, the cells grown in both of the flexible flasks and T-175 flasks were harvested by trypsinization and the cell yields from each culture vessel were determined by calculating the number of stained nuclei within 30～45 minutes after collection. Trypan blue exclusion method was employed to determine the cell viability.

2.5. Homogeneity of cell growth distribution

In order to observe homogeneity of cell growth distribution, a crystal violet staining method was applied (Abraham et al., 2011). At the end of culture, the medium in both of the flexible flask and T-175 flask was carefully removed and the cells were rinsed twice with PBS. To visualize the distribution of attached cells by naked eye, the cells were fixed by 10% methanol (Analytical reagent, Dengke chemical Reagent Co. Ltd. Tianjin, China) for 20 minutes and stained with 0.1% crystal violet solution for 10 minutes followed with distilled water washing to remove residual dye. After removing the water and drying, the whole bottom of the flask and the bioreactor was photographed.

2.6. Statistic analysis

All the experiments were repeated at least three times and the statistic analysis software SPSS 13.0 was used for the data analysis. The data was shown in mean ± standard deviation value of the three parallel sets of experiments. Statistical significance was accepted for a value of P <0.05.

3. Results

3.1. The structure feature and stability of the flexible flask

As shown in Figure1-2, the flexible flask was made of a single sheet of plastic film by folding and thermo-compress fusion techniques. Like a rigid flask, the flexible flask possessed a three-dimensional (3D) configuration with five sides, one bottom, two quadrangle side walls and two triangle side walls, supporting each other. At the edges of two adjacent side walls, the film was fused together to form a fusion edge, which, like a backbone frame, played an important role in keeping a freestanding 3D configuration. In order to reduce cost during transportation and storage, the flexible flask could be folded as shown in Figure 2b.

3.2. Evaluation of Cell attachment

In order to study the cell attachment capacity of the PP/SEBS material of the inner surface of the flexible flask compared with TC-treated T-flask, morphology observation and quantitative analysis of attached cells were performed at different time points. Although the roof of the flexible flask was not flat, it could be easily placed on the platform of the microscope and observed any sites of the bottom by pressing the top edge down to change the flatness of side walls. Benefit from the extreme transparency of the film, the cell morphology could be observed under the microscope clearly. As shown in Figure 3, Vero cells gradually settled down on the surface in the culture vessels after inoculation. During adherent process, the cells underwent a morphological change from a round shape to a spreading form with filopodia, which were the same as that observed in T-flasks. At 24 hours post seeding, cell attachment rate achieved 88.1 % and 90.0% in the flexible flasks and T-flasks, respectively. However, the quantitative analysis of adherent cells (Figure 4) showed that adherent rates in flexible flasks were compatible with that in T-flask (P＞0.05). The same results were obtained in the culture of 293A cells.

3.3. Cell yield and Cell viability

Considering that Vero cells and 293A cells both well attached to the film. The data shown in the figure was based on Vero cells growth. All the cultured cells grown in flexible flasks and T-flasks reached near complete confluence after 48 hours growth. The cell yield obtained from the flexible flasks showed no significant difference with that of the T-175 flasks ($4.18\pm0.50\times10^5$ cells ml-1 vs. $4.34\pm0.14\times10^5$ cells ml^{-1}, P＞0.05). The cell viability in the flexible flask was comparable with that in T-175 flask (98.02±0.85% vs. 98.84±0.69) without significant difference (P＞0.05). In the enlarged flask with a scale equivalent to three T-175 flasks, the cell yield increased proportionally and similar cell viability were obtained, also no statistic significance in the two indicators between the two kinds of culture vessels (data not shown).

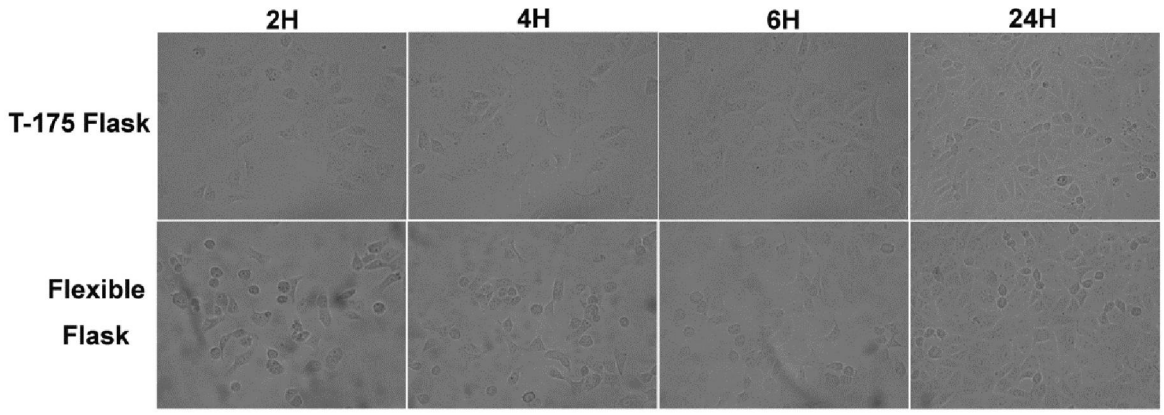

Figure 3. Photographs of adherent cells at different time point (100×)

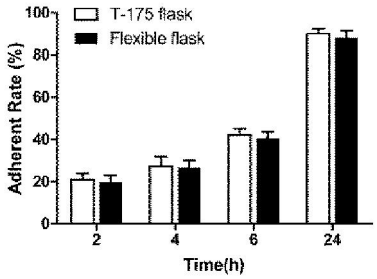

Figure 4. Assessment of attachment efficiency of Vero cells grown in these vessels

3.4. Cell homogenous distribution

The cells stained with crystal violet showed that cell attachment and growth on the bottom of γ-irradiated the flexible flask was well homogenous and similar to that on the flat bottom of TC-treated T-175 flask (Figure 5).

Figure 5. Representative photographs of distribution of Vero cells grown in the two vessels

4. Discussions and Conclusion

Rigid single layer T-flask is commonly used for static cell culture at laboratory scale with many advantages, such as easy to handle and convenient to observe under a phase contrast microscope. However, to enlarge the culture surface and keep it flat for either single layer or multi-layer T-flasks is challenging and strict art demanding by using such rigid material from a manufacturing standpoint. Due to non-foldable, the rigid hollow flask with a large internal space occupied a large portion of the total flask volume for accommodation of gas leads to the increased cost for manufacturing, shipping and storage. Therefore, making flexible culture vessels should be an ideal alternative.

Guyre et al. (2002) reported the application of Life cell bags made of gas permeable film in static culture of adherent cells. It was a closed two-dimensional (2D) bag by laminating two sheets of film together and formed a pouch-like structure after filling medium. However, it could not keep a 3D internal space by itself and is unsuitable to collect cells by scrapping for Western analysis. In this study, we developed a pentahedron shaped flask which enabled to keep itself a freestanding 3D internal space without any other supports. The film used for making the flexible flask was a kind of multiple-layer co-extrude medical plastic film and possessed many advantages, including exceptionally clear, gas and liquid impermeable, tough sturdy, chemically inert, high tensile and abrasion resistance. As the Figure 3 shown, Vero cells could well attach to and grow on the bottom surface of the inner layer PP/SEBS material of the flask, which achieved compatible effects with the rigid plastic flask in cell attachment, viability, yield and distribution. The crystal violet staining result demonstrated a homogenous distribution of cells grown on the bottom of the flexible flasks were observed (Figure 5) and suggested that the inner surface of the film is a favorable material for anchored cell growth.

Due to the flexibility of the film, to keep the flexible flask with 3D internal space is a great challenge and thus we designed the flexible flask in a triangular shape in its cross section based on the stability of a triangular structure. It is the triangular

structure together with the fusion edges that enabled the flexible flask to keep its 3D internal space steadily without needing air inflation and any other supports. Also, it could be folded (seen Figure 2b) to save room and reduce cost during shipping and storage. As we know, to make an oversized T-flask and keep its bottom flat is a high-tech demanding manufacture art, whereas to manufacture a flexible flask with a flat bottom, especially oversized, is simple and easy by taking advantage of the flexibility of the film. With this film, we were able to easily make different sized flexible flasks as desired, for example, an enlarged flask with a scale equivalent to three T-175 flasks was developed as shown in Figure 2a and a proportional increase in the cell yield (data not shown) was acquired. Though the enlarged flask used a single vent cap same as that of T-175 flask, the cell yield demonstrated the vent cap could provide enough aeration for cell growth in a scale of at least three T-175 flasks.

Like rigid flasks, the flexible flask could be handled easily, such as pipetting medium into or out from the flask (Figure 2c-e) or collecting cells by scrapping easily any bottom sites of the flask through adjusting the angles of the rigid pipe. However, it is inconvenient or impossible to perform such operations in multi-layer flasks or cell factories.

Though the roof of the flask wasn't flat, it was able to perform cell morphological observation at any spot under a phase-contrast microscope by pressing the top edge down. Also, the angles between the quadrangle side walls and the bottom was as small as possible (usually less than 45 degrees) under the principle to keep enough space for accommodation of air. Just as the multi-layer T-flask, its main purpose is to amplify bio-products, not for morphological observation.

Since the amount of medium for static culture of adherent cells is usually small, the flexible flasks at size below 175 cm^2 were not caused obvious deformation during transfer process. For the oversized ones, deformation of the flexible flask might be caused by the gravity of large amount of medium during transfer, but, if happened, the deformed flask could be easily recovered by manually stretching the side fusion edges. For transportation convenience, application of a transparent flat plate to support the bottom of the bioreactor, such as a glass or a poly-carbonate plate was preferable so as to prevent any possible deformation of the flask and cell detachment.

In conclusion, the novel flexible flask was an ideal alternative culture vessel of rigid plastic T-flask for static culture of adherent cells at laboratory scale with advantages, such as foldable, easy to scale up, convenient to operation and less waste generation.

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