# *In Vitro* Evaluation of Cell Loss: Retention and Repopulation on Substrates upon Shear Flow by a Rheometer

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## Abstract

In this work, we utilized a cone and plate type rheometer to assess the adhesive force between bovine carotid arteries endothelial cells and substrate materials. The cell retention and repopulation on these materials were observed in vitro. The results showed that the rheometer was a convenient tool to evaluate adhesive force and cell retention on a biomaterial substrate. On gelatin-coated surface, cells were more resistant to shear flow compared to those on tissue culture polystyrene or biocompatible poly(carbonate)urethane. The newly grown cells on the gelatin substrate after flushing for a period of time became further resistant to shear flow.

***Keywords***: Rheometer, Shear flow, Adhesive force, Cell retention, Cell repopulation

## Introduction

The long-term success of prosthetic vascular grafts has been limited primarily to larger grafts with diameters of > 6 mm. This limitation is due in large part to the frequency of luminal surfaces on the grafts to activate components of the coagulation system. For grafts with diameters smaller than 6 mm, this results in a high incidence of implant occlusion [1].

Endothelial cells line the luminal surface of all elements of the vascular system. These cells exhibit numerous metabolic functions necessary for the maintenance of homeostasis. The critical role of endothelium in maintaining normal blood vessel function is exemplified by the poor clinical performance of small diameter polymeric vascular grafts which fail due, in part, to the lack of a functional endothelium on the luminal surface [2]. However, efforts of seeding endothelial cells on prosthetic materials could be done only with little success because endothelial cells detached with ease under shear flow condition on materials in vivo [3]. Thus, to find the hemocompatible materials with endothelial cells attaching firmly on them is a very important goal in cardiovascular tissue engineering.

The cone and plate type rheometer (cone angle < 5°) is a well-defined rheological system in which a homogeneous laminar flow is generated by a rotating cone or plate. In the cone and plate type rheometer, a small volume of medium is enough to load the material surface with constant shear stress [4-6]. In the current investigation, we utilized the cone and plate type rheometer as a tool to assess the adhesive force between bovine carotid arteries endothelial cells and substrate materials. The substrate materials in the study included: synthesized poly(carbonate)urethane, a common substrate of vascular grafts; gelatin, a common sealant for such grafts; and the tissue culture polystyrene as the control. The cell loss, retention and re-endothelialization on these three materials under the shear flow condition were observed in vitro.

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## Materials and Methods

### *Cell culture*

Bovine carotid arteries endothelial cells (BEC) were obtained as previously described [7]. They were cultured in a 25cm$^2$ tissue culture flask using Dubelcco's modified Eagle medium (DMEM) with 10% (v/v) fetal bovine serum and 1% (v/v) Penicillin-Streptomycin. The cells were incubated in 5% $CO_2$-95% air at 37°C.

### *Preparation of materials*

Tissue culture polystyrene (TCPS) control dish (FALCON®) with a diameter 51mm, gelatin-coated dish, as well as poly(carbonate)urethane (PCU) were the substrates investigated in this study. To prepare the gelatin-coated dish, 5% gelatin solution was coated onto the dish and was crosslinked for 2 days by 1% 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride dissolved in 40% alcohol solution. After crosslinking, the gelatin dishes were immersed in distilled water for 2 days to remove any residual crosslinking agent. PCU was synthesized by condensation polymerization of 4, 4-diphenylmethane diisocyanate (MDI),

poly(hexyl, ethyl)carbonate diols (PC diol), and *cis*-2-butene-1, 4-diol (BDO) in N, N-dimethyl acetamide at 60°C by two-step polymerization. The molar ratio of MDI/PC diol/BDO in the polymerization recipe was 3/2/1. The PCU thin films were prepared by casting the PCU solution on the polytetrafluoroethylene(PTFE) plate. The solvent was removed by a vaccum oven. The dried films were then peeled from the plate and pasted on the tissue culture dish. All the materials were sterilized by 70% alcohol solution and then washed carefully by phosphate buffered saline before use.

***Cell seeding***

Confluent BEC were detached from tissue culture flask with 0.05% trypsin-0.53mM EDTA solution, transferred to DMEM medium and centrifuged (180g for 5min at room temperature). The resultant pellet was resuspended in growth medium and a cell inoculum $5\times10^4$ or $1\times10^5$ cells/cm$^2$ introduced into each dish.

The seeded dishes were divided into two groups and cultured in fresh growth medium for either 24 hours or 48 hours before flow testing.

***Application of shear flow***

A controlled-strain rheometer (RFS-8500E) was utilized to apply a steady shear stress on the endothelial cells cultured on the surface of tissue culture dishes. This system was based on a cone and plate type rheometer. The cone was made of titanium, with parameters 50 mm in diameter, and 0.02 radian in cone angle. About 700 μl of culture medium was loaded onto the material surface in the rheometer (Figure 1). Continuous shear stress of 11 dyne/cm$^2$ was applied to endothelial cells for one hour for each exposure. The interval between each shear exposure was one day. A total of three exposures were conducted in three days at 37°C. Between each shear experiment, the dishes were replaced with fresh culture medium and cultured in an incubator.

***Cell staining and observation***

The remnant endothelial cells on the dishes were stained by Safranin O to determine the cell distribution after the shear flow. The stained cells were examined by an inverted phase contrast microscope (Nikon TE-300, Japan).

## Results

When the seeding density was $5\times10^4$ cells/cm$^2$ and the time for cell adhesion was 24 hours, cells on all substrates were flushed away upon exposure to the shear flow for one hour. Therefore, a seeding density $1\times10^5$ cells/cm$^2$ was selected. The time allowed for cell adhesion before starting the flushing experiment was 48 hours instead.

After the first exposure to the shear flow for one hour, most cells on TCPS or PCU were lost (Figure 2(a),(b)). The results obtained for TCPS and PCU were very similar, so only the data of TCPS were shown in figures. The cells remained on these substrates were more spread in the morphology. On the gelatin substrate, only a part of the cells were removed by the flow (Figure 2(c),(d)). The cell morphology on gelatin before and after the first shear exposure was alike.

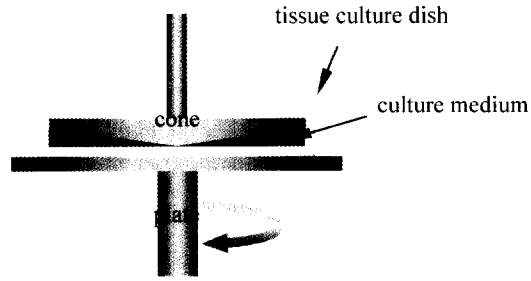


Figure 1. Configuration of a cone and plate type rheometer.

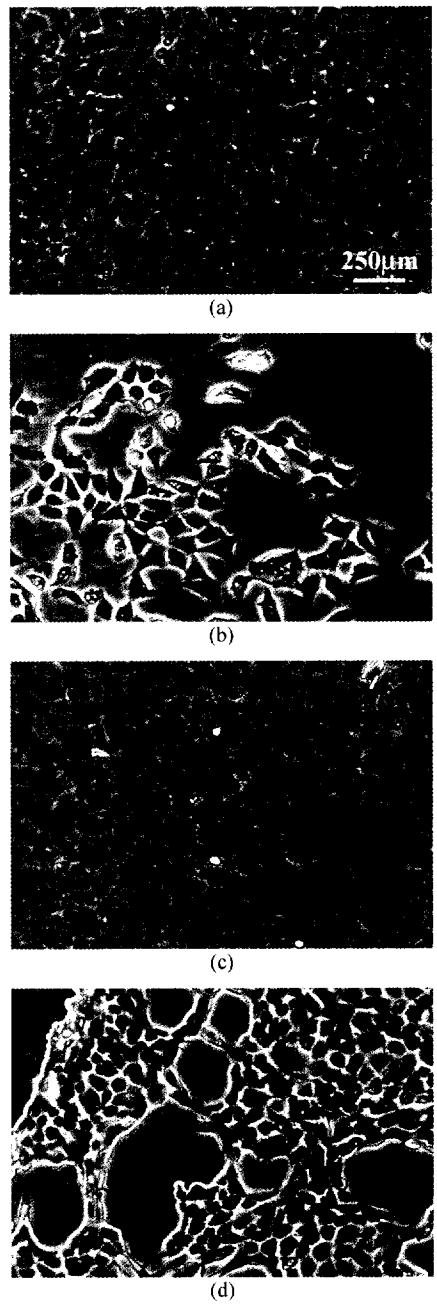


Figure 2. Phase contrast microphotographs showing cells on TCPS and gelatin before and after the 1st exposure of shear stress for one hour. (a) TCPS/before;(b) TCPS/after;(c) Gelatin/before;(d) Gelatin/after.

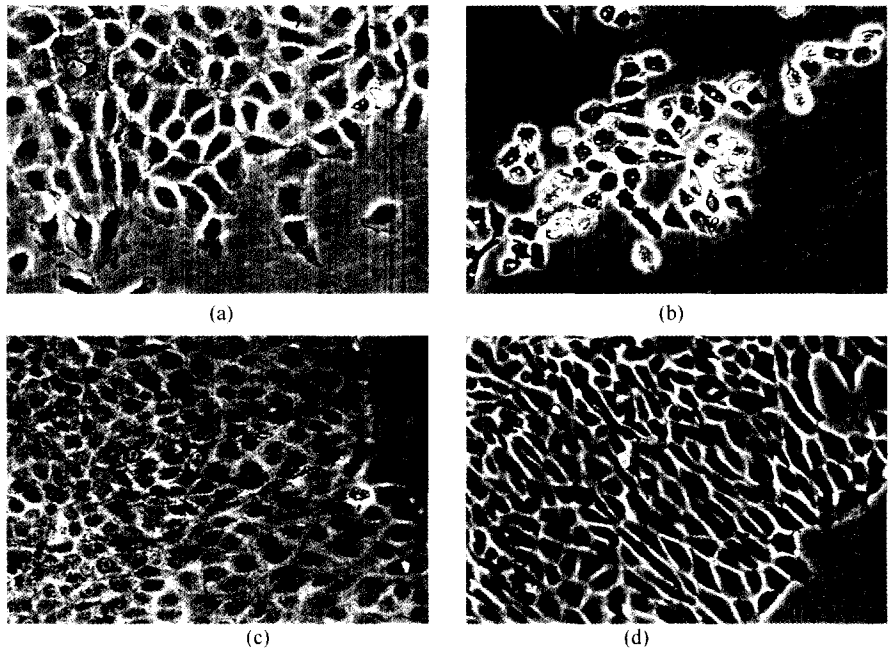


Figure 3. Phase contrast microphotographs showing cells on TCPS and gelatin before and after the 2nd exposure of shear stress for one hour. (a) TCPS/before; (b) TCPS/after; (c) Gelatin/before; (d) Gelatin/after.

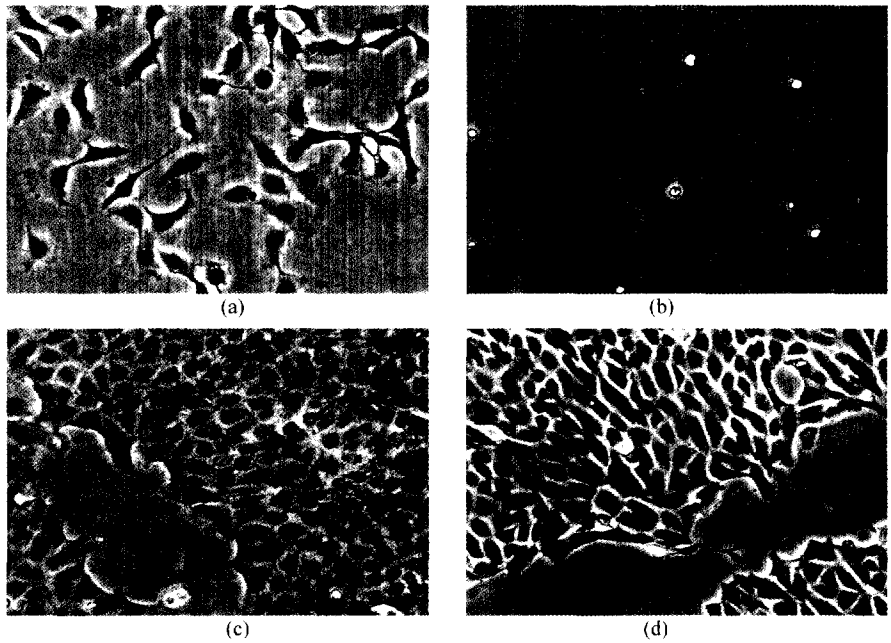


Figure 4. Phase contrast microphotographs showing cells on TCPS and gelatin before and after the 3rd exposure of shear stress for one hour. (a) TCPS/before; (b) TCPS/after; (c) Gelatin/before; (d) Gelatin/after.

Before the second exposure to the shear flow on the next day, there were newly grown cells on all substrates. Generally, cells became larger in size due to the reduced crowdedness in space (Figure 3(a),(c)). After another hour of shear exposure, the cells on TCPS or PCU were lost again, similar to that observed on the pervious day (Figure 3(b)). However, cells on gelatin demonstrated a better resistance and most cells still remained on the substrate (Figure 3(d)). By examining carefully, the cells on gelatin substrate became elongated and were aligned in a direction perpendicular to the tangent of the rotational plate.

Before the third exposure, cells on TCPS and PCU were highly spread in morphology (Figure 4(a)). All cells were completely lost after one hour of shear experiment (Figure 4(b)). On the other hand, the cells on gelatin before shearing were less oriented (Figure 4(c)). Cells remained attached on the gelatin upon shear exposure, yet again showing more elongation and alignment (Figure 4(d)).

The macroscopic observation of the cell distribution on different substrates was achieved by Safranin O staining and is shown in Figure 5. The loss of cells from TCPS or PCU surface continued from the first shear exposure to the third

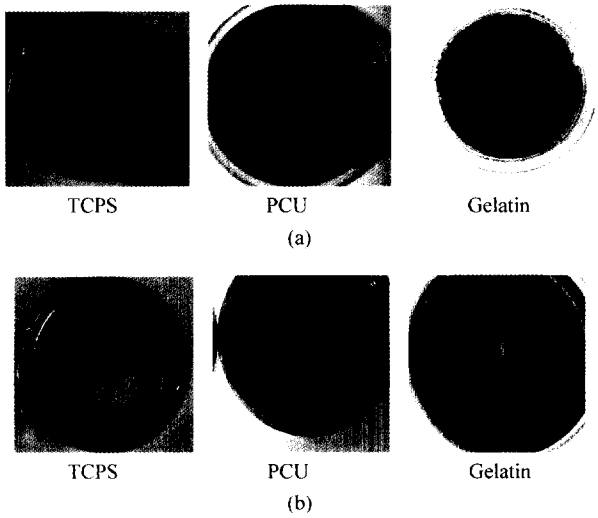


Figure 5. Macroscopic view of cell distribution on different substrates after the 1st and the 3rd exposures. (a) After first exposure; (b) After third exposure. Almost all cells were lost from TCPS and PCU after the 3rd exposure. Cells repopulated the gelatin substrate after the 3rd exposure.

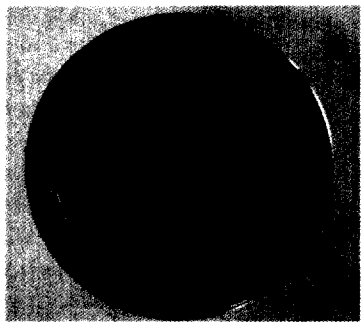

Figure 6. Macroscopic view of cell distribution after zero operation in the rheometer.

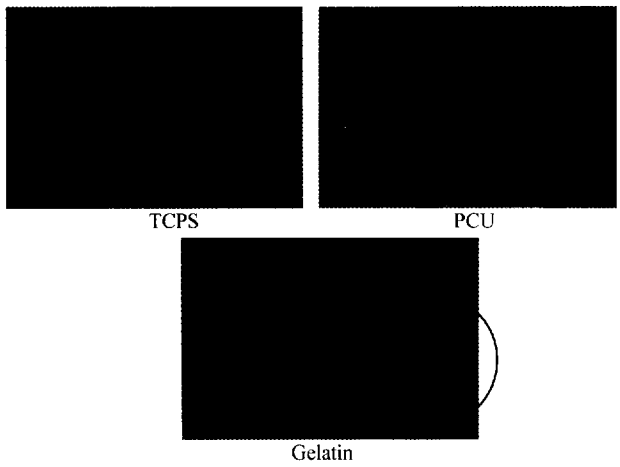


Figure 7. Phase contrast microphotographs showing extracellular matrix (ECM) remaining only on the gelatin surface after cells were removed by the exposure of flow (3rd exposure). Circle indicates the area of ECM residue.

exposure. Hardly any cells were present on TCPS or PCU after the third exposure. On the contrary, more cells were observed on gelatin after the third exposure than on gelatin after the first exposure. This indicated that the gelatin substrate was repopulated with cells upon repeated exposures to shear stress.

We also found that the standard operation of "zeroing" the rheometer created a central defect area where the cells were injured before the shear experiment. The cells started to detach from the defect area during the shear experiment, as shown in Figure 6.

The substrate after each shear exposure was examined carefully for the trace of extraceullar matrix secreted by the cells. Residue of extracellular matrix on gelatin but not on the other two substrates was observed after shear experiment, as demonstrated in Figure 7.

## Discussion

In evaluating the endothelial cell retention, an in vitro perfusion system with cells seeded tubes flushed with the serum-free medium at 37°C and a flow rate of 220 ml/min that produced a wall shear stress of 20 dyne/cm$^2$, or a similar setup, was often employed [8]. This method of evaluation was time-consuming. In addition, seeding procedures and cell counting were complicated in such a perfusion system. Using flow chambers or a rheometer to assess the adhesive force of endothelial cells was a simpler way. Furukawa et al [3] have used a cone and plate rheometer to evaluate the cell detachment from the substrate. In order to detach the cells efficiently, the shear stress used in their study was 100 dyne/cm$^2$, which was much greater than the physiological stress condition. Under such a large stress, cell repopulation was probably slow and could not be readily studied. Therefore, we proposed to use a physiological shear stress in this study. Besides, repopulation of cells could also be evaluated using the same system.

The effect of shear stress on the signal transduction and gene expression in endothelial cells has been extensively studied by Chien et al [9] and others. Nevertheless, the effect of shear on the cell alignment was less studied. Kataoka et al [10] used a flow chamber to study the morphological response of cultured bovine aortic endothelial cells to flow direction, and observed most of the cells elongated and aligned with the flow direction (i.e. parallel to the shear stress). On the other hand, Wang et al [11] applied a cyclic strain to endothelial cells cultured on a silicone membrane and observed that endothelial cells reoriented in the direction of minimal substrate deformation (i.e. vertical direction to the strain). In our study, cell alignment was also observed. This observation agrees with the previous two reports. Since it was the plate rather than the cone that rotated in our rheometer setup, a centrifugal force was generated on cells. This is probably why the cells appeared to align in the direction of the centrifugal force (i.e. perpendicular to the rotational contour).

There is also disadvantage of using a rheometer in assessing the cell retention. Since the cone has to touch the cells to make the zero calibration during the operation, cells near the center were damaged unavoidably. Dead cells formed

an aggregate that scrubbed the nearby surface and caused a subsequent loss of the cells around them. This is why a small circle of cell loss was observed in the center of all exposed dishes.

We believe that the increased resistance of the newly grown cells on gelatin was attributed to the secretion of the extracellular matrix (ECM) and the retention of these substances on the gelatin upon the shear flow. Thoumine et al [12] observed an increase amount of laminin and other ECM secreted by cultured endothelial cells exposed to laminar flow. Laminin and other ECM deposited on the substrate, however, could be flushed away by the shear. Unlike the smooth TCPS or PCU surface, crosslinked gelatin substrate was highly swollen and could retain the ECM within its network. The accumulation of ECM after repeated shear exposures probably helped hold the endothelial cells on the gelatin substrate. The repopulation of endothelial cells on a substrate was thus dependent on the natures of the substrate, e.g. ability to preserve extracellular matrix secreted by the cells.

## Conclusion

We concluded that using a rheometer was a simple and convenient way to assess the cell loss, retention and repopulation on material substrates. Upon repeated shear exposures, endothelial cells repopulated on gelatin-coated dishes and remained firmly attached. The cell response to the shear may depend on the nature of the substrates. Adequate shear and substrate material could promote proliferation and health condition of cells, as well as retain extracellular matrix for cells and protect cells from the further shear. Based on these, gelatin was probably a preferred surface for re-endothelialization.

## Acknowlegment

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## References

[1]. H. P. Greisler, "Growth factor release from vascular grafts", *Journal of Controlled Release*, 39: 267-280, 1996.

[2]. S. K. Williams, "Endothelial cell transplantation", *Cell Transplantation*, 4: 401-410, 1995.

[3]. K. S. Furukawa, T. Ushida, T. Nagase, H. Nakamigawa, T. Noguchi, T. Tamaki, J. Tanaka and T. Tateishi, "Quantitative analysis of cell detachment by shear stress", *Materials Science and Engineering C*, 17: 55-58, 2001.

[4]. A. Bhagyalakshmi, F. Berthiaume and K. M. Reich, "Fluid shear stress stimulates membrane phospholipids metabolism in cultured human endothelial cells", *Journal of Vascular Research*, 29: 443, 1992.

[5]. K. S. Furukawa, T. Ushida, H. Sugano, N. Ohshima and T. Tateishi, "Real time observation of platelet adhesion to opaque biomaterial surfaces under shear flow conditions", *Journal of Biomedical Materials Research*, 46: 93-102, 1999.

[6]. K. S. Furukawa, T. Ushida, H. Sugano, T. Tamaki, N. Ohshima and T. Tateishi, "Effect of shear stress on platelet adhesion to expanded polytetrafluoroethylene, a silicone sheet, and an endothelial cell monolayer", *ASAIO Journal*, 46: 696-701, 2000.

[7]. H. Hagiwara, M. Shimonaka, M. Morisaki, N. Ikekawa, Y. Inada, "Sitosterol-stimulative production of plasminogen activator in cultured endothelial cells from bovine carotid artery", *Thrombus Research*, 33: 363-370, 1984.

[8]. K. C. Kent, A. Oshima, T. Ikemoto, A. D. Whittemore, "An in vitro model for human endothelial cell seeding of a small diameter vascular graft", *ASAIO Transactions*, 34: 578-580, 1988.

[9]. S. Chien, S. Li, J. Y-J. Shyy, "Effects of mechanical forces on signal transduction and gene expression in endothelial cells", *Hypertension*, 31: 162-169, 1998.

[10]. N. Kataoka, S. Ujita, M. Sato, "Morphological response of cultured bovine arotic endothelial cells to flow direction", *Biorheology*, 32: 113-114, 1995.

[11]. J. H. Wang, P. Goldschmidt-Clermont, J. Wille, F. C. Yin, "Specificity of endothelial cell reorientation in response to cyclic mechanical stretching", *Journal of Biomechanics*, 34: 1563-1572, 2001.

[12]. O. Thoumine, R. M. Nerem, P. R. Girard PR, "Changes in organization and composition of the extracellular matrix underlying cultured endothelial cells exposed to laminar steady shear stress", *Laboratory Investigation*, 73: 565-576, 1995.

# 利用流變儀評估體外流場下細胞在基材上之殘存與長回

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## 摘 要

在此研究中，我們使用錐與盤型之流變儀，來評估牛頸動脈內皮細胞對基材之附著能力。在流變儀施予體外流場之後，可觀察細胞在基材上之殘存率與其後再長回之情形。結果發現，流變儀確爲評估生醫材料上細胞之附著力與流場下細胞在基材上殘存率的有利工具。與組織培養盤之聚苯乙烯或生物相容性之聚碳酸酯型聚胺酯這兩種材料相比，在塗佈有明膠的基材上，細胞具有較佳之抵抗流場剪應力之能力，而且，其後新長回之細胞，較原先之細胞，更能抵抗流場的進一步沖刷。

**關鍵詞**：流變儀、剪流、附著力、細胞殘存、細胞長回

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