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# In vitro study of wound-healing capabilities of bioactive glass fibers under various culture conditions

Sisi Chen

# IN VITRO STUDY OF WOUND-HEALING CAPABILITIES OF BIOACTIVE GLASS FIBERS UNDER VARIOUS CULTURE CONDITIONS

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SISI CHEN

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Yinfa Ma, Advisor
Honglan Shi, Co-advisor
Klaus Woelk

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## ABSTRACT

Bioactive borate glass has been recognized to have both hard and soft tissue repair and regeneration capabilities through stimulating both osteogenesis and angiogenesis. However, the underlying physiological and cellular mechanism behind this function remains unclear. In this study, in vitro dynamic flow modules were designed to mimic the micro-environment near the vascular depletion and hyperplasia area in wound-healing regions, and were used to investigate the biocompatibility and functionality of borate glass nano-/micro-fibers. Glass-cell interactions were investigated either by dosing fibers to the upstream of or co-cultured with cells, and two types of borate glasses (with or without CuO/ZnO doped were compared with a silicate-based one. The results showed substantial dissolution of fibers in the cell medium, with both elemental ions (boron, sodium and potassium) releasing and calcium/phosphate deposition onto fiber residues reflected by SEM images and EDS analysis. Positive effects revealed by better mitochondrial activity were observed on cells treated with three types of fibers, and cells exposed to the borate copper/zinc containing 1605 fibers were found to have the most salutary influence. Meanwhile, the results demonstrated that 1605 can better stimulate VEGF secretion over the other two with dual-chamber configuration at 0.5 mL/h and with single-chamber configuration at 1.0 mL/h. These results may indicate a potentially significant mechanism of wound-healing with regard to angiogenesis enhancement.

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**PAPER**

# Stimulated Secretion of Vascular Endothelial Growth Factor (VEGF) by Bioactive Borate Glass Nano-/Micro- Fibers under Dynamic Conditions


## ABSTRACT

Bioactive borate glass has been recognized to have both hard and soft tissue repair and regeneration capabilities through stimulating both osteogenesis and angiogenesis. However, the underlying physiological and cellular mechanism behind this function remains unclear. In this study, *in vitro* dynamic flow modules were designed to mimic the micro-environment near the vascular depletion and hyperplasia area in wound-healing regions, and were used to investigate the biocompatibility and functionality of borate glass nano-/micro-fibers. Glass-cell interactions were investigated either by dosing fibers to the upstream of or co-cultured with cells, and two types of borate glasses (with or without CuO/ZnO doped were compared with a silicate-based one. The results showed substantial dissolution of fibers in the cell medium, with both elemental ions (boron, sodium and potassium) releasing and calcium/phosphate deposition onto fiber residues reflected by SEM images and EDS analysis. Positive effects revealed by better mitochondrial activity were observed on cells treated with three types of fibers, and cells exposed to the borate copper/zinc containing 1605 fibers were found to have the most salutary influence. Meanwhile, the results demonstrated that 1605 can better stimulate VEGF secretion over the other two with dual-chamber configuration at 0.5 mL/h and with single-chamber configuration at 1.0 mL/h. These results may indicate a potentially significant mechanism of wound-healing with regard to angiogenesis enhancement.

**Keywords:** Bioactive borate glass fibers, Cu/Zn doped glass fibers, vascular endothelial growth factor (VEGF) secretion, *in vitro* dynamic flow design, wound healing

## 1. Introduction

Wound healing is a dynamic and interactive process that involves four coordinated stages that involve angiogenesis: acute inflammation, re-epithelialization, granulation tissue formation, and tissue remodeling [1-3]. This process is initiated immediately after injury by releasing various biological molecules. Many growth factors are involved in the physiological angiogenesis [4-6] that associated with wound healing [7], among which the vascular endothelial growth factor (VEGF) is a prominent one [4, 8] for stimulating wound healing by enhancing epithelialization and collagen deposition [8, 9]. VEGF functions as an endothelial cell mitogen [10], cell migration and proliferation [11], and inducer of vascular permeability [12]. Therefore, VEGF is an essential component in wound healing and its role has been studied to understand the underlying mechanism [9].

Despite the fact that the healing process is triggered immediately after an injury and all wounds go through similar phases of repair, non-healing wounds or large areas of skin impairment raise people's concerns [1]. Current approaches to promote wound healing include applying artificial skin substitutes, such as epidermal and dermal skin substitutes, however, they suffer from limitation of availability, control of infection [1]. Self-healing materials composed of microencapsulated healing agents have been reported to demonstrate decent mechanical performance and regenerative ability [13], but they fail in autonomic repair of injury in a given location [14].

Bioactive glasses have been shown to promote osteogenesis, which has inspired novel uses for both hard and soft tissue engineering [15-17]. Compared with other biomaterials, such as wound care dressing, bioactive glass has demonstrated high biocompatibility. For example, fast degradation to hydroxyapatite (HA) and flexible shape make them a candidate for tissue engineering[18]. After developed by Hench et al. in 1970 [19], the silicate-based bioactive glass (45S5) has become the most widely used bioactive glass composition among others in both scientific research and clinical applications [16, 20]. The *in vivo* glass transformation processes, including the formation of hydroxyapatite (HA), is extremely important for bioactive glass related osteogenesis. However, the relatively slow degradation and conversion rate of 45S5 bioactive glass have limited its clinical applicability [21, 22]. Thus the efforts to develop new bioactive glass formulations with more rapid degradation/conversion rates and better wound-healing effect have expanded in recent years.

Bioactive glass was enabled to be specifically tailored with desirable compositions. Borate-based glass, as one of the new types of bioactive material, has been used in bone repair, drug delivery [23-25] with promising functions. Its positive effect on cell proliferation [22] and angiogenesis [26] further renders it as potential candidate for soft-tissue repair and regeneration [26, 27]. The bioactive ion releasing and hydroxyapatite (HA) conversion were recognized as the major mechanism that underlying wound-healing capabilities [28]. This process can significantly be enhanced in borate-based glasses than their silicate-based counterparts [21], suggesting a potentially better effect of these materials. Similarly, other types of glass composition tailoring may profoundly influence the ion releasing pattern of the materials, thus impact the

interactions between materials and biological systems. For example, copper ions were found to stimulate the proliferation of human endothelial and osteoblast-like cells [29, 30], promote angiogenesis [31, 32], and stimulate VEGF secretion [33], which are essential steps during wound healing. Also, zinc ions are utilized by many metalloenzymes for structure, catalytic or regulatory actions [34]. Both of these ions, and plus calcium, phosphorus, silicon and magnesium, etc., can be correlated with angiogenesis and many bioactivities that related to wound-healing [28], and thus should be considered as specific glass tailoring components for better bioactive effects.

However, boron (and other metal elements) does have its own cytotoxicity concerns under a normal static co-culturing condition with cells. High concentration of $B_2O_3$ has been reported to produce a greater inhibition of cell proliferation [22]. On the contrary, some preliminary *in vitro* and *in vivo* experiments that used such materials, showed more or less positive physiological responses of cells, by adopting a type of dynamic culturing environment [22, 26, 35]. As a result, the negative effects that observed under relatively static culturing conditions were mostly attributed to disrupted local pH [21]. Therefore, conclusions that draw under static conditions may not fully address the potential bioactivity of the borate-based glasses. In this case, the *in vitro* reproducing of a dynamic condition that closely mimic in-body environment becomes critical for function investigation of new bioactive materials.

In this study, a home-built dynamic flow system was designed and fabricated to better investigate the interactions between bioactive glass and cultured cells. A human skin fibroblast cell line CCL-110 was used to carry out VEGF secretion tests. One silicate-based (45S5) and two borate-based (13-93B3 and 1605) bioactive glass fibers

were employed and compared in their effect on cell growth and a major growth factor (VEGF) secretion under either non-contact mode or in-contact mode, depending on glass fibers were dosed separately or together with cultured cells.

## 2. Materials and methods

### *2.1. Bioactive glass fibers*

Three different compositions of bioactive glass fibers were obtained from Mo-Sci, Inc. (Rolla, MO, USA) in the form of cotton-like bundles of discontinuous fibers, 50 nm-2 μm in diameter. Glass formulations studied included a silicate-based glass designated **45S5** (45% $SiO_2$, 24.5%$Na_2O$, 24.5% CaO, 6%$P_2O_5$, wt%), and two borate glasses; **13-93B3** has a nominal composition of (6% $Na_2O$, 12% $K_2O$, 5% MgO, 20% CaO, 4% $P_2O_5$, and 53% $B_2O_3$ wt%) and **1605** has the same nominal composition except that 1.4 wt% $B_2O_3$ was replaced by 0.4 wt% CuO and 1 wt% ZnO.

### *2.2. Dynamic Flow Module Fabrication*

The dynamic flow module reaction chambers were made with Dow Corning Sylgard 184 Silicone Elastomer (Polydimethylsiloxane, PDMS) according to the manufacturer's instructions (Ellsworth Adhesives Company, Germantown, WI). The elastomer is a two-part liquid, silicone elastomer base and silicone elastomer curing agent mixed at a 10:1 ratio. About 30 mL of mixture was placed into an evacuated vessel to degas, then transferred to a glass mold, was after which it was cured in an oven at 70 °C for 60 minutes and then removed from the mold. Two types of reaction chambers were produced: the dual chamber configuration (**Scheme 1a**) was used to separate the nanofibers from the cell colony whereas the single chamber configuration (**Scheme 1b**)

was used for experiments where the fibers and cells were in contact. The total volume of the chambers in both design configurations was about 0.6 mL. Along with the connection between two chambers, the volume is around 1 mL. Fibers were weighed with a micro-balance (5g capacity and 1 μg resolution, ME 5, Data Weighing Systems, Inc., Elk Grove, IL), and a paste was made by mixing 1 mg of fiber with 1 mL of water. This paste was then immediately transferred to the reactor to initiate a dynamic flow experiment (described in section 2.4).

*2.3. Cell line and culture conditions*

The human fibroblast cell line CCL-110 was obtained from American Type Culture Collection (ATCC) (Manassas, Virginia) with standard Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) (Life Technology, Grand Island, New York), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 50 U/ml penicillin and 50 mg/ml streptomycin. Cells were cultured at 37 °C with 5% $CO_2$ and 95% humidity.

*2.4. Cell and bioactive glass fiber imaging*

Cell mitochondria specific dye JC-1 (Life Technology, Grand Island, New York) and cell nucleus staining dye 4', 6-diamidino-2-phenylindole (DAPI) (Life Technology, Grand Island, New York) were used to enhance image contrast. JC-1 solution was prepared in dimethyl sulfoxide (DMSO) at a stock concentration of 1 mg/mL and was diluted 500 fold for staining cells. DAPI solution was diluted 300 fold for final use. Cells were stained with JC-1 for 60 minutes and 15 minutes with DAPI before taking images. Three fluorescent channels of DAPI (excitation 355 nm, emission 465 nm), FITC

(excitation 488 nm, emission 525 nm) and Cy5 (excitation 488 nm, emission 590 nm) were separately taken under microscope (Olympus IX51, Pennsylvania) and the respective images were merged together.

Fibers were collected with 0.22 μm nylon membrane filters after soaking in cell culture medium for 72 hours under the dynamic flow conditions. Fresh and soaked fibers were imaged by field emission scanning electron microscopy (FESEM) (Hitachi 4700, Tokyo, Japan) and characterized by energy-dispersive X-ray spectroscopy (EDS) (FEI, Hillsboro, OR, USA).

*2.5. In vitro cell proliferation*

Human skin fibroblast cells CCL-110 were allowed to grow to 75%-80% confluence in 24 hours prior to each cell test. To determine the cell proliferation, the reagent WST-1(Roche Life Science, Indianapolis, Indiana) was used following the manufacturer's instructions. The cells were exposed to bioactive glass fibers for 24, 48, and 72 hours at dosing concentration of 50 μg/mL, 100 μg/mL, 500 μg/mL, and 2000 μg/mL. Absorbance at 450 nm using WST-1 was measured using a microplate reader (FLOURstar; BMG Labtechnologies, Durham, North Carolina).

*2.6. Dynamic Experiments*

For the experiments where nanofibers and cells were separated, the cells were pre-seeded ($5 \times 10^4$ cells/cm$^2$) for 16-18 hours in the down-stream chamber of dual chamber configuration **(Scheme 1a**). An initial mass of 1 mg of bioactive glass fibers was then added to the up-stream chamber and a constant flow of fresh culture medium was provided via a syringe pump. An empty up-stream chamber without dosing fibers

was served as control. Similar experimental procedures were used in the fiber-cell contact experiments, except that the fibers were added to the same chamber with the pre-seeded cells **(Scheme 1b**). For the initial dynamic experiments using both chamber configurations, the flow rate was kept constant at 0.5 mL/h for 72 hours. Solution samples were collected at prescribed times, filtered and stored at -20 °C before analysis. A higher flow rate (1.0 mL/h) experiment was also conducted using the single-chamber configuration, with all other experimental variables remaining the same. The flow rates were chosen based on the previous study[36], the calculated amount of blood through skin or subcutaneous tissue is around 74 mL/day (3 mL/hour). Therefore, two flow rates were studied in this work (0.5 mL/h and 1 mL/h). The studied flow rates were closed to the *in vivo* conditions in order to approach to the real situation.

*2.7. ICP-OES analysis of ions released from the bioactive glass fibers*

The concentrations of ions released from the bioactive glass fibers during the dynamic flow experiments were measured by inductively coupled plasma - optical emission spectrometry (ICP-OES) using a model 2000DV inductively coupled plasma - optical emission spectrometer (PerkinElmer, Massachusetts, USA). Samples collected at different times were filtered (0.22 μm nylon membrane filter) and diluted with 1% $HNO_3$ for ICP-OES analysis. Elements were analyzed at 208.89 nm (B), 214.94 nm (P), 251.61 nm (Si), 285.21 nm (Mg), and 317.93 nm (Ca), Na- and K-ion releases were not monitored because the high concentrations of these elements in the cell culture medium made it impossible to reproducibly detect their small increases in concentrations when the glasses dissolved. The quality control tests including instrument calibration, reagent

blank, sample replicate, and spiked recovery were all performed before and during sample analysis to ensure the data quality.

*2.8. Measurements of VEGF secretion and total protein*

VEGF secretions were quantified from a 50 µL volume from each sample using VEGF Human enzyme-linked immunosorbent assay (ELISA) kits (Life Technology, Grand Island, New York) as manufacturer's instructions. The optical density of each sample was measured with a FluoStar microplate reader (BMG Labtech Inc., Ortenberg, Germany) at a wavelength of 450 nm. Samples collected at 3 hours, 24 hours, 48 hours, and 72 hours were tested. The total protein concentrations were measured with Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA) according to instructions. The bovine serum albumin was used as a standard and the samples were measured with a UV-vis detector at wavelength of 595 nm.

*2.9. Statistical analysis*

Experimental data were analyzed by one-way ANOVA followed by Post Hoc test. All experimental results are expressed as the mean and standard deviation (SD) based on triplicate measurements. The level of statistical significance was presented as a *p*-value of $<0.05$ (*).

## 3. Results

*3.1. Bioactive glass nanofiber characterization*

After soaking for 72 hours, the morphological and elemental changes of all fibers were characterized by FESEM (**Fig. 1**). Highly roughened surfaces were mostly observed in all three types of fiber. However, more fragments and fiber pieces were

observed in borate-based fibers after soaking. Elemental analysis using EDS showed significant decreases in the relative intensities of the Na and K signals, and increases in the relative intensities of the Ca and P signals, however, boron was not detected. The Ca/P atomic ratios of the 45S5, 13-93B3 and 1605 fibers were similar (**Table 1**). Compared with Ca/P ratio before soaking, the ratio of 45S5 fibers did not decrease significantly. However, this ratio decreased from 9.0 to 6.4 and 11.1 to 6.4 of 13-93B3 and 1605, respectively.

*3.2. In vitro cell proliferation*

Cell proliferation assays were conducted using fresh bioactive glass fibers at different dosages and varied testing times. Four concentrations were tested at a 72-hour exposure time (**Fig. 2**). At 50 μg/mL, 45S5 exhibited a positive effect on cell proliferation while 13-93B3 and 1605 showed insignificant influences on cell growth. As the dosage increased, the negative effects of fibers became dominant, especially when concentration was 2000 μg/mL. Among different fibers, 1605 showed the most harmful impact on cell proliferation compared with control group where cells did not expose to fibers.

*3.3. Major ion release kinetics*

A series of time lapse ion-releasing kinetic assays were carried out using the home-built dynamic flow module. The dual-chamber configuration was firstly used, and followed by the single-chamber one. Along with a constant culture medium flow, the effluents of both control and fiber-dosed samples were collected with specified time intervals. ICP-OES analyzed ion concentrations were then plotted after subtraction of the

control data points, thus revealed the actual elemental releasing patterns (**Fig. 3**). For the dual-chamber configuration, a releasing peak was observed for most ions within first 8 hours, followed by quick concentration reduction (**Fig. 3a**). Phosphorus concentration decreased significantly in all fiber-dosed samples, and its final concentration was lower than the original level. While with the single-chamber configuration, where cells and fibers were co-cultured, the phosphorus concentration in effluents varied when given different flow rate. A lower flow rate (0.5 mL/h) presented no negative reduction of phosphorus and calcium ions (**Fig. 3b**) while higher flow rate (1 mL/h) showed the negative values (**Fig. 3c**). The continuous negative reductions of phosphorus and calcium were noticed with all fiber types in dual-chamber configuration and single-chamber configuration at high flow rate (**Fig. 3a and 3c**). With single-chamber configuration, the releasing peak where high flow rate (1.0 mL/h) provided appeared to be sharper than that of low flow rate (0.5 mL/h) (**Fig. 3b and 3c**). Si ions were only detected with single-chamber configuration at high flow rate (0.5 mL/h) for the purpose of confirmation. Nevertheless, the total concentrations of released ions were found out to be the same.

*3.4.Mitochondria activity assessment*

Our previous study showed potentially positive effect of these bioactive fibers on cell viability and migration[35] when co-cultured under certain dynamic flow conditions, thus a mitochondria activity assay for 72 hours was designed to correlate cellular energy consuming with dynamically-soaked fibers. A mitochondria inner membrane specific dye JC-1 was used in the single-chamber configuration, and both Cy5 and FITC channel images were taken and compared (in fluorescent intensity) quantitatively, plus with DAPI channel images taken for cell number confirmation (**Fig. 4)**. Cell nucleus was well

maintained throughout co-culturing, indicating no major cell damage was caused by the glass fibers. For up to 72 hours, cell mitochondria in control group (without fiber exposure) showed excellent condition as demonstrated by high red & low green fluorescence feature. However, green fluorescence appeared in all three fiber-dosing groups after 36 hours, and was continued to 72 hours, which indicating a certain degree of mitochondrial depolarization was occurred. A following-up quantitative measurements of fluorescent intensities using ImageJ software showed an gradually increasing trend (see median line in each boxplot) of the fluorescent intensity in Cy5 channel in all three fiber dosed groups, whereas a slightly decreasing trend in the control group (**Fig. 5**). Thus the ratio between red and green fluorescent intensities varied alongside the dosing time. At 36-hour point, 45S5 fibers showed slightly higher $ratio_{cy5/FITC}$, while after 72 hours, borate fibers showed higher averaged $ratio_{cy5/FITC}$ than control and the 45S5 fiber groups, with statistically significant difference between the 1605 fiber and control groups ($p < 0.05$). This increase indicated a possible lower mitochondrial depolarization rate and thus, a healthier cell condition after fiber exposure.

Since direct cell viability assay was not applicable under a constant dynamic flow condition, total protein analysis was thus carried out to indirectly evaluate cell growth conditions (**Fig. 6).** The single-chamber configuration was also applied in this test, and after 72 hours, the total cellular protein was determined using Bradford method[37]. Results showed a decreased cellular protein levels in groups that with glass fiber exposure. Compared to the control, about 30% lower, and even 50% lower proteins were respectively observed, in groups that treated with silicate- and borate-based fibers. This result indicated fewer cell mass were presented after 72 hours fiber-exposure.

*3.5.VEGF secretion*

In order to further analyze the impact of fiber-exposure to cell growth ability, the VEGF secretion level was monitored. Both dual- and single-chamber configurations were applied, and different flow rates were compared using the single-chamber configuration (**Fig. 7**). Levels of VEGF secretion gradually increased in the dual-chamber configuration, with fiber-type dependent influences (**Fig. 7a**). The VEGF concentration increase by 45.2 pg/mL, 49.8 pg/mL, and 62.2 pg/mL corresponding to dosages of 45S5, 13-93B3 and 1605 after 72-hour exposure. The 1605 fibers at 48 hours, and both the borated-based fibers at 72 hours, had stimulated VEGF secretion significantly ($p < 0.05$), compared with the 45S5 fibers and control. But with same flow rate, the VEGF secretion was not stimulated as much when using the single-chamber module configuration. After doubling of the dynamic flow rate (from 0.5 mL/h to 1 mL/h), however, the secreted VEGF came up to a higher level, especially featured by the significant increasing that stimulated by fiber 1605 after 48 and 72 hours exposure (**Fig. 7c**). The VEGF concentrations increased by 24.9 pg/mL and 106 pg/mL after exposure to 1605 at flow rate of 0.5 mL/h and 1.0 mL/h, respectively.

## 4. Discussion

Bioactive materials, including the recently developed fiber-shaped glasses, have shown significant potentialities to promote bone regeneration and tissue repair [38-40]. However, many previous studies were performed under static condition [41, 42] which might not be able to adequately reproduce a real biological circumstances, and might also overrate the cytotoxic effect by the materials [43]. The present *in vitro* study is

unprecedentedly based on a fine dynamic control system that closely approached the *in vivo* wounded areas, which can work superiorly as standard procedure for bioactive material evaluation.

Our results showed both morphological and compositional changes of the bioactive glass fibers after soaking, and should be attributed to comprehensive reactions between fiber surface and species within medium [16]. Particularly, released Ca-ions would preferably react with surrounding phosphate anions and form a calcium-phosphate complexity that possibly deposited onto the fiber surface (**Table 1**). The Ca/P ratio of stoichiometric HA layer is 1.67 [44]. Such ratio within the soaked fibers did not reach that level presumably due to the short contact time compared with published study (7 days) [26]. Thus it may appear in other forms and still affect VEGF secretion that observed in the later results. However, the Ca/P deposition was proved by the following ion releasing assay where both species showed a negative final concentration in collected effluents.

A much faster ion releasing pattern was observed in this study compared to a previously showed result that investigated under static condition [35]. This changed ion releasing rate may well impact the surrounding cell growth status differently. For example, boron was reported to have toxic effect on cell proliferation [45, 46], but the dynamic flow as well as the dual-chamber configuration may highly reduce the local borate concentrations and thus decreases its cytotoxicity [43, 46]. In this case, mechanism interpretation under a simulated dynamic condition should provide a better understanding than previous experimental set-ups that mostly using a static infrastructure. Besides, when considering biomaterial dissolving under a dynamic rather than a static

environment, many concentration-dependent bioactivities need to be taken into consideration as well. Taking boron as an example again, it is an essential element for bone health and its effect on tissue growth appears to be concentration dependent [47], large quantity is toxic, but trace amount can promote osteoblast proliferation [48]. Thus the synthetic formula of bioactive glass materials and their compatibility under biologically relevant dynamic flow conditions are extremely important for wound healing effects [26, 49]. The angiogenic potential related factor can well be evaluated by measuring one of the growth factors, like VEGF , as a surrogate biomarker due to its critical function [8]. The results (**Fig. 7)** showed that all three types of bioactive glass fibers played a positive role in VEGF secretion by fibroblast cells. Previous studies have shown that 45S5 can stimulate the expression and release of VEGF and bFGF [41], whereas this study demonstrated that the borate-based bioactive glasses, 13-93B3 and 1605, have enhanced the capability for promoting VEGF secretion under dynamic flow conditions. Particularly, $Cu^{2+}$ ions was shown to stimulate the proliferation of human umbilical vein endothelial cells at concentrations around 32 μg/mL [29]. Other reports recently showed that $Cu^{2+}$ ions can promote the production of angiogenic factors, including VEGF [26, 32, 50]. Another important element, zinc and its dissolved form, $Zn^{2+}$ ions, are involved in a variety of physiological processes including cell proliferation as well [51]. These reports are consistent with the present observation that the 1605 glass fibers produced optimal VEGF secretion among the glass formulations studied herein.

Another issue that needs further consideration is the indirect or direct fiber-cell contact. In the current study, cell proliferation and activity was examined under both indirect and direct contact modes. Ions released from the fibers played the dominant role

in fiber-cell interactions, while the direct contact mode may embrace more comprehensive reactions, including the morphological and compositional changes of the fiber surfaces, and local pH and ion concentration gradients. Our results indicated a better VEGF stimulation by silicate-based fibers in cell-fiber contact mode under a relatively lower flow rate (**Fig. 7b**), whereas the borate-based 1605 glass fibers stimulated VEGF secretion at greater rates under the higher flow rate conditions (**Fig. 7c**). The local pH change may also modulate the cell behavior [52, 53], however a higher flow conditions will induce less surrounding pH changes. Finally, the calcium-phosphate layer that formed on the fiber surface may play a key role as well [54] (**Fig. 1, Table 1**). The conversion rates of borate-based bioactive glass fibers in phosphate solutions to calcium phosphate are faster than those of silicate-based bioactive glass fibers [21]. Thus accordingly, more rapid formation of a calcium phosphate layer on the 1605 fibers may contribute to the impact on the growth factor secretion.

## 5. Conclusion

The present study provided information that demonstrated borate-based bioactive glass fibers can stimulate VEGF secretion from human fibroblast cells under dynamic flow conditions. Two dynamic *in vitro* modules with different configurations were developed and applied in current studies. Soaking fibers in cell culture medium led to the morphological and compositional changes and the formation of calcium-phosphate layer on fiber surfaces. The ions released from the fibers into cell culture medium, such as Ca, P, and B, were effective on VEGF stimulation. Moreover, the fibers also play a role in VEGF secretion. While borate-based fibers are capable of improving VEGF secretion at low flow rate (0.5 mL/h), the stimulation was significantly enhanced at high flow rate

(1.0 mL/h). The mitochondrial statuses demonstrated cells were more health after exposure to bioactive glass fibers. Overall, the results indicated that borate-based bioactive glass fibers were not toxic to human fibroblast cells, and the current designed dynamic *in vitro* system not only enable us to simulate *in vivo* conditions of the interactions between bioactive glass fibers and human fibroblast cells, it also provided a platform for further study on wound healing mechanism of bioactive materials and for the optimization of bio-active glass compositions to further improve bio-functionality.

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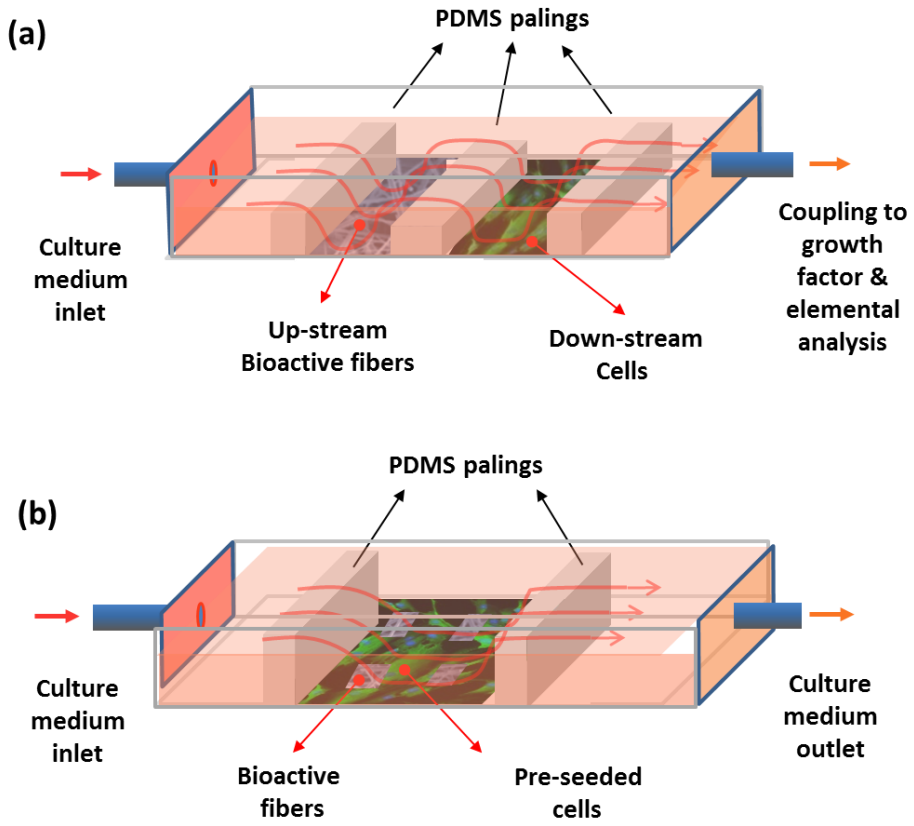
Table 1. EDS analysis of the Ca- and P-concentrations of fiber surfaces before and after a 72-hour dynamic soaking experiments.

| | **45S5** | | **13-93B3** | | **1605** | |
|---|---|---|---|---|---|---|
| | Fresh | Soaked | Fresh | Soaked | Fresh | Soaked |
| **Ca (At%)** | 9.5 | 11.3 | 9.9 | 13.9 | 8.9 | 17.1 |
| **P (At%)** | 1.5 | 4.1 | 1.1 | 5.4 | 0.8 | 5.9 |
| **Ca/P ratio** | 6.3 | 2.8 | 9.0 | 2.6 | 11.1 | 2.9 |




Scheme 1. Dynamic modules made with PDMS. (a) dual-chamber configuration, cell-fiber separation mode; (b) single-chamber configuration, cell-fiber contact mode.

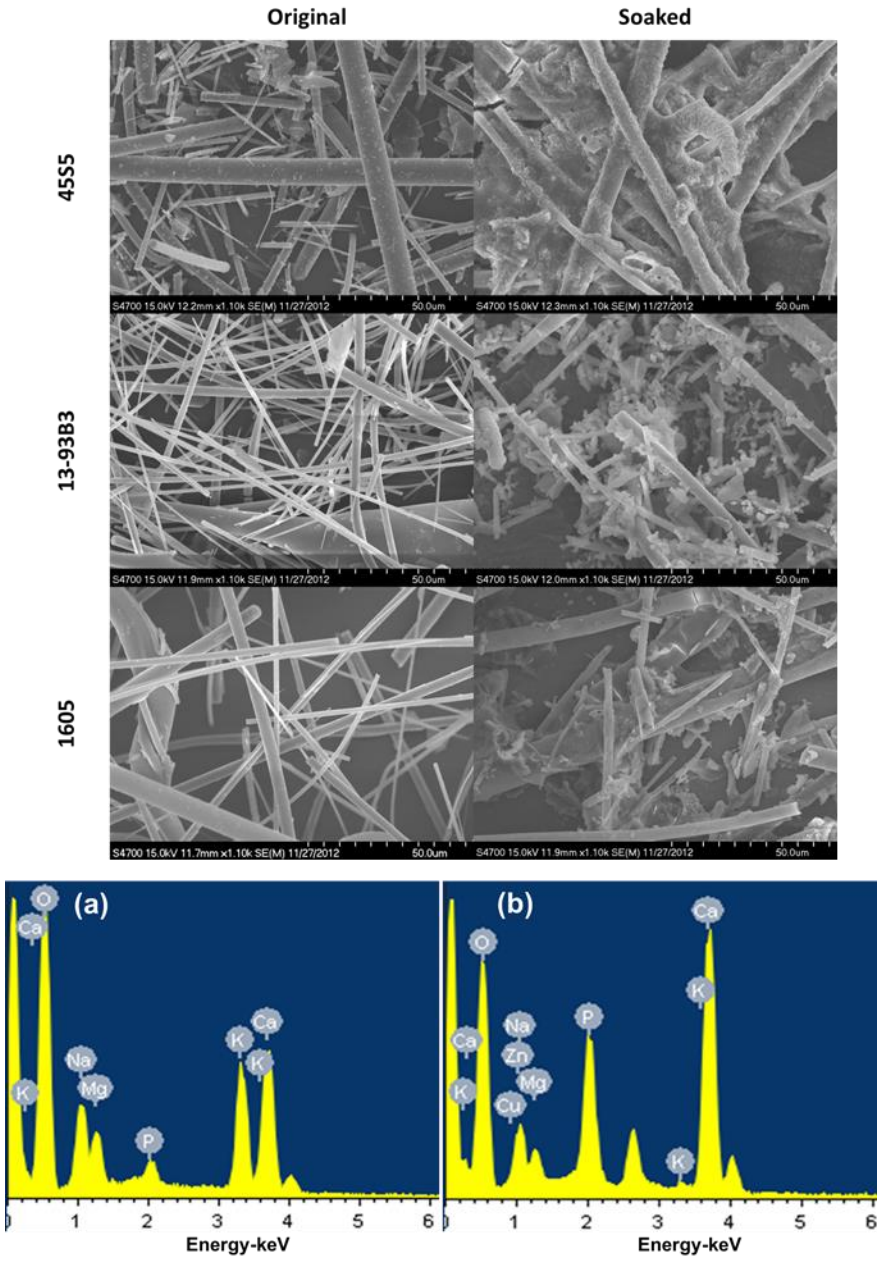



Fig. 1. SEM images and EDS scanning of bioactive glass fibers before (a) and after 72 hours (b) soaking in cell culture medium under dynamic condition.

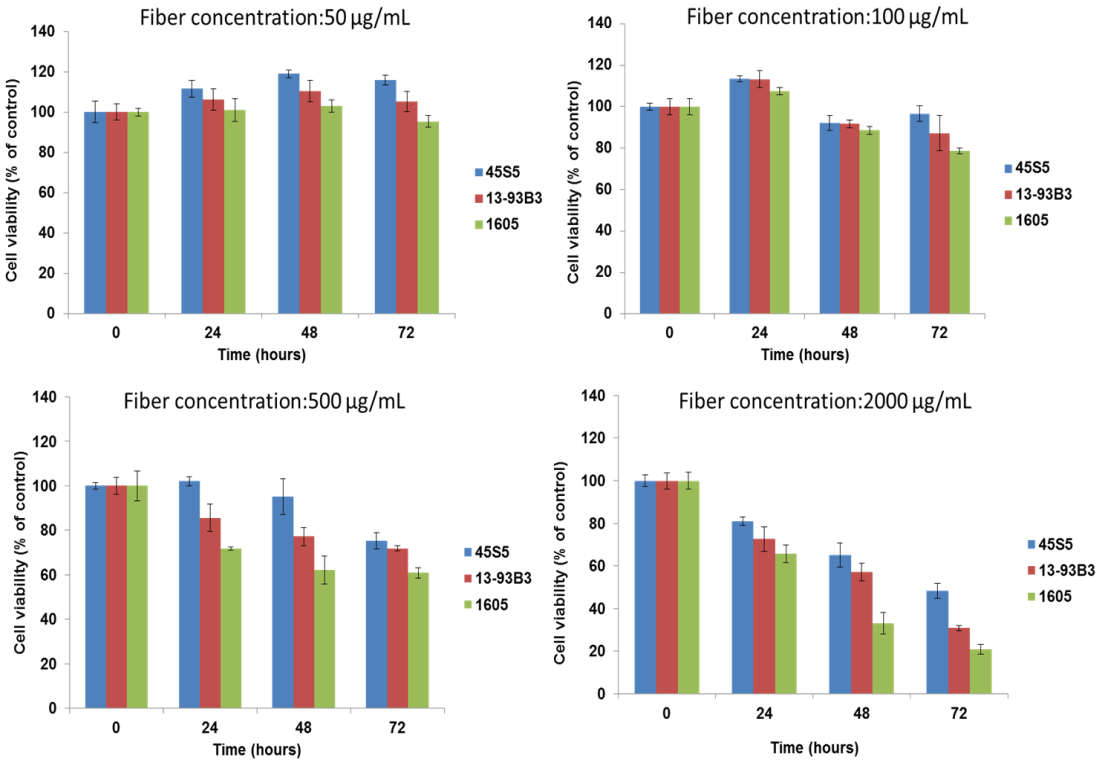



Fig. 2. Cell proliferation assays under static culture conditions. Cells were treated with 45S5, 13-93B3, and 1605 for 24, 48, and 72 hours, respectively. Values are mean ± SD (n=3).

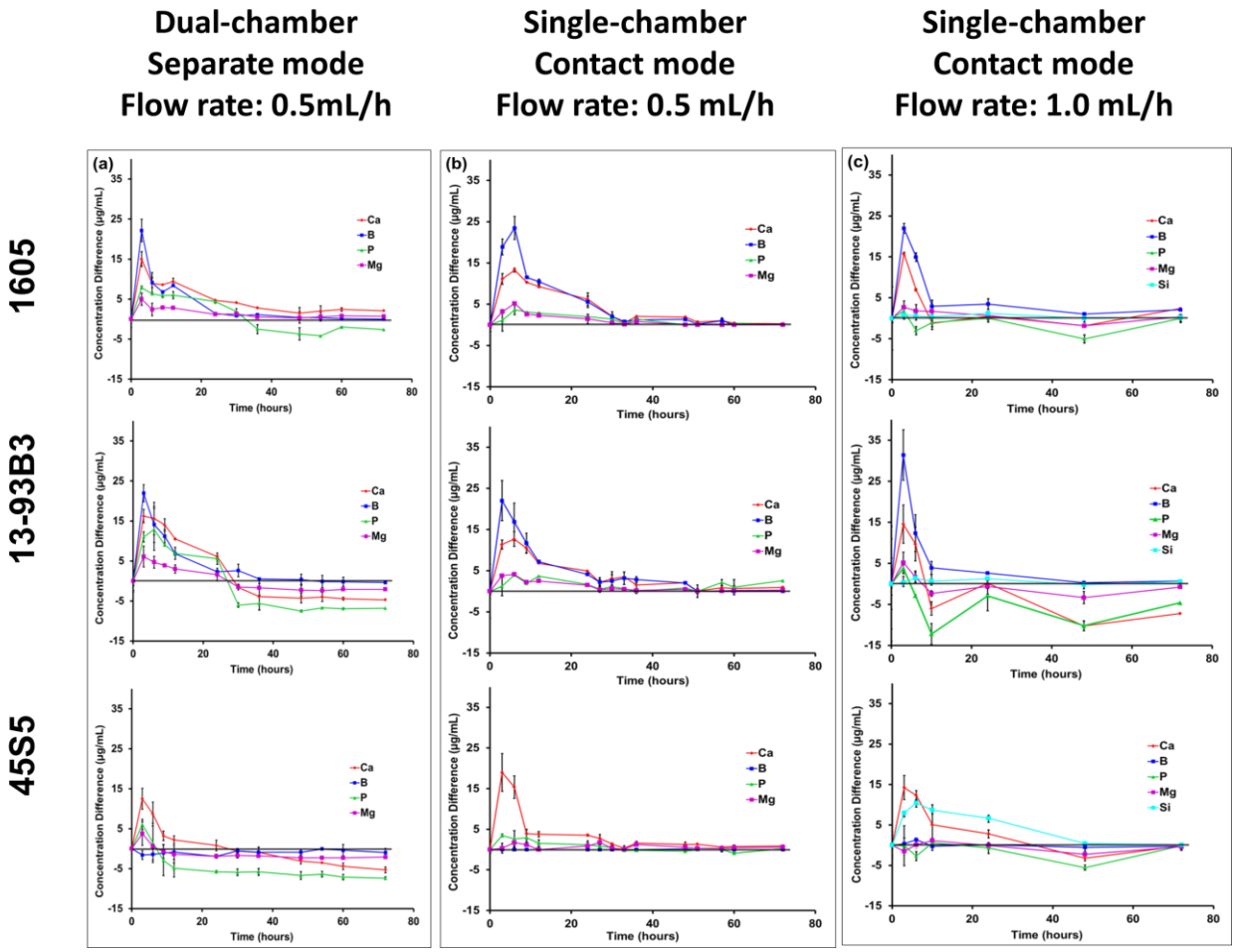



Fig. 3. Normalized major ion release kinetics in dynamic experiments. The concentrations are presented as the differences between the sample concentrations and the control (cell culture medium) concentrations. (a) with dual-chamber configuration, flow rate at 0.5 mL/h; (b) with single-chamber configuration, flow rate at 0.5 mL/h; (c) with single-chamber configuration, flow rate at 1.0 mL/h.

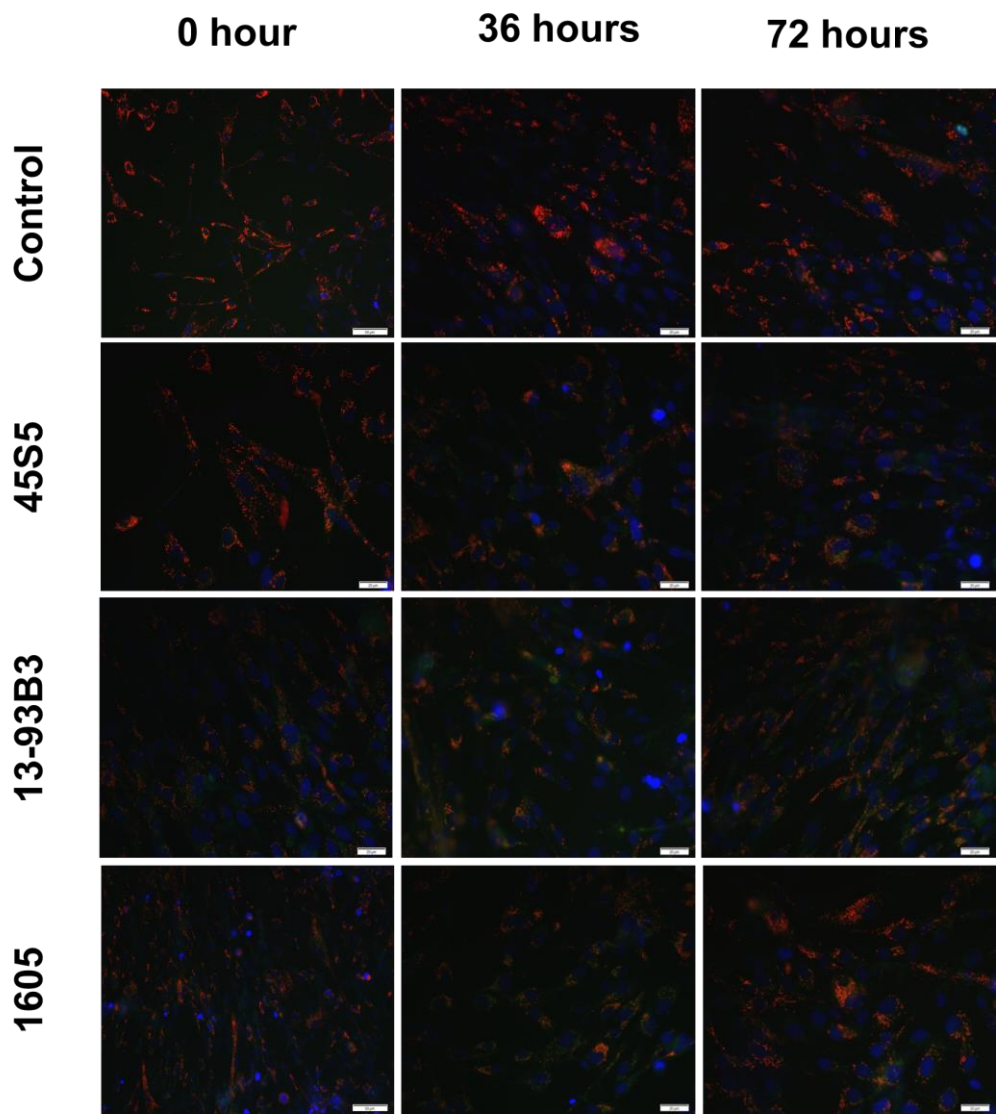



Fig. 4. Cell images of control (no fibers), 45S5, 13-93B3 and 1605 at 36 hours and 72 hours contact time in a single-chamber (cell-fiber contact) experiment at 1.0 mL/h cell culture medium flow rate.

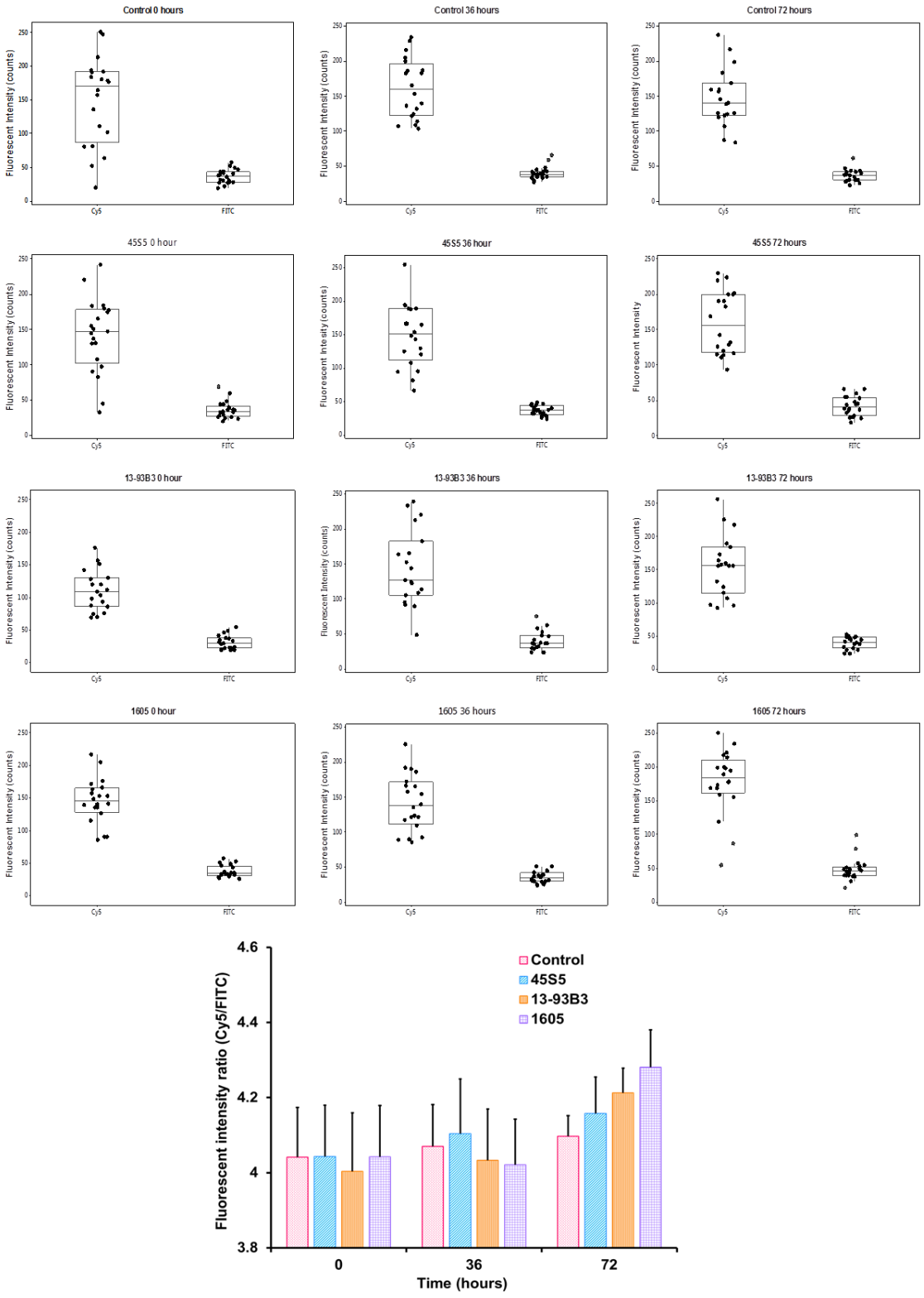



Fig. 5. Statistical analysis of mitochondrial staining with JC-1 dye of control and fiber treated cells at 0, 36, and 72 hours in single-chamber experiment at 1.0 mL/h cell culture medium flow rate.

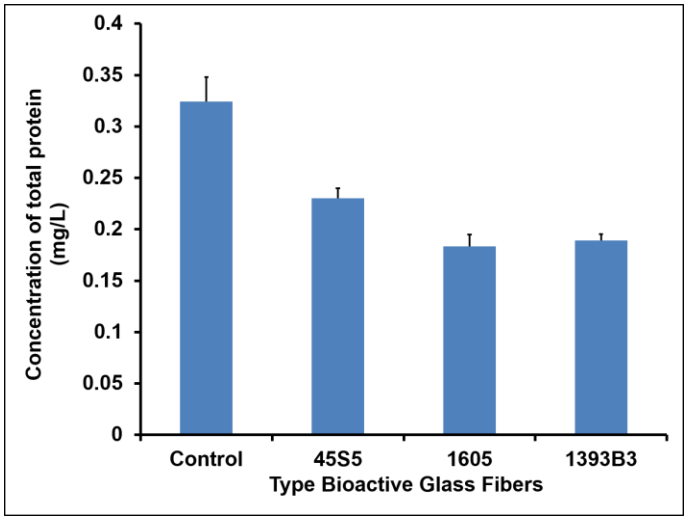



Fig. 6. Total protein concentration measurements of three bioactive glass nanofibers along with control after 72-hour experiment where cells and fibers were in contact at flow rate of 1 mL/h.

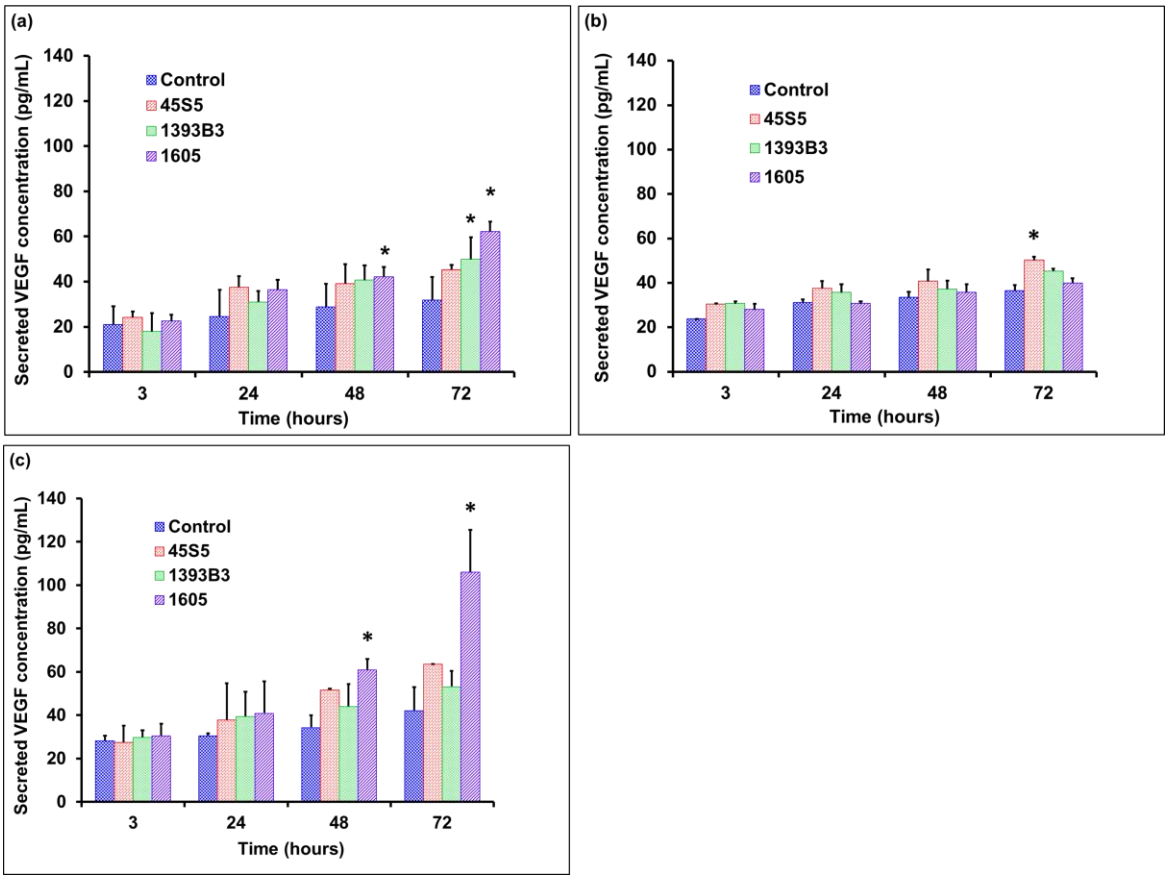



Fig. 7. VEGF secretion by human fibroblast cells. (a) dynamic double-chamber tests with a flow rate of the cell medium of 0.5mL/h; (b) dynamic single chamber dynamic tests with a flow rate of the cell medium at 0.5mL/h; (c) dynamic single chamber dynamic tests with a flow rate of the cell medium at 1.0mL/h. Significance indicated by $*p < 0.05$ versus control cells.

# VITA

Sisi Chen was born on October 10, 1989 in Beijing, China. She completed her primary and secondary education in Beijing, China. In 2008, she was admitted by School of Chemical Biology and Pharmaceutical Sciences, Capital Medical University, Beijing, China. After she received her BS degree in 2012, she joined Missouri University of Science and Technology to continue her study in Dr. Yinfa Ma's research group. She received her Master of Science in Chemistry from the Missouri University of Science and Technology in December, 2015.