Nanonet Force Microscopy for Measuring Forces in Single Smooth Muscle Cells of Human Aorta

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Abbreviations:
STEP: Spinneret based Tunable Engineered Parameters
NFM: Nanonet Force Microscopy
ECM: Extracellular Matrix
SMC: Smooth Muscle Cell
I-O: Inside Out
O-I: Outside In
ROS: reactive oxygen species

ABSTRACT
A number of innovative methods exist to measure cell-matrix adhesive forces, which have yet to accurately describe and quantify the intricate interplay of a cell and its fibrous extracellular matrix (ECM). In cardiovascular pathologies, such as aortic aneurysm, new knowledge on the involvement of cell-matrix forces could lead to elucidation of disease mechanisms. To better understand this dynamics we measured primary human aortic single smooth muscle cell (SMC) forces using nanonet force microscopy (NFM) in both inside-out (I-O intrinsic contractility) and outside-in (O-I external perturbation) modes. For SMC populations, we calibrated the I-O and O-I forces to be 12.9±1.0 and 57.9±2.5 nN respectively. Subsequently, exposure of cells to oxidative stress conditions caused a force decrease of 57% and 48% in I-O and O-I modes respectively and an increase in migration rate by 2.5fold. Finally, in O-I mode, we cyclically perturbed cells at constant strain with varying duration to simulate in vivo conditions of the cardiac cycle and report I-O forces to reduce with increasing duration, while the O-I forces dropped by half at shorter cycle times. Thus, our findings highlight the need to study forces exerted and felt by cells simultaneously to comprehensively understand force modulation in cardiovascular disease.

INTRODUCTION
Smooth muscle cells (SMCs) receive mechanical and chemical stimuli from the extracellular matrix (ECM) via integrin mediated focal adhesions (Moiseeva, 2001). For a vascular SMC, this interaction plays an important role in modulating vascular resistance and tone, thereby affecting the resistance of a vessel. SMCs generate forces via...
actomyosin contractions, which impart a mechanical force on the surrounding ECM (Gunst and Zhang, 2008). This leads to vasoconstriction or dilatation of vessels, affecting overall systemic vascular resistance. Furthermore, in the arterial system, particularly in the aorta, there is an ECM directed force generated by contraction in the cardiac cycle, which is experienced by the SMCs. The pulsatility causes the collagen and elastin microarchitecture to stretch and the resulting stretch force is transmitted through the focal adhesions to the cytoskeletal network.

Establishing a contextually relevant fibrous platform to understand cell generated (inside-out, I-O) and ECM generated (outside-in, O-I) forces is integral to the study of disease states. At the tissue level, for example, characteristic histopathological features defining the pathophysiology of ascending thoracic aortic aneurysms include degeneration of the elastin matrix, non-inflammatory loss of SMCs, and biomechanical weakening of the aortic wall (Nataatmadja et al., 2003). In this regard, our previous work has revealed distinctions between the aneurysmal patient cohorts and from non-aneurysmal, healthy aortic specimens at the cell, ECM, and tissue levels. Specifically, aneurysmal aortic specimens exhibited higher tensile strength, lower delamination strength, anisotropic collagen and elastin fiber microarchitecture and SMCs were more susceptible to oxidative stress when compared to SMCs from degenerative aneurysm specimens and non-aneurysmal aortas (Phillippi et al., 2009b, 2010, 2014; Pichamuthu et al., 2013; Tsamis et al., 2014).

At the single-cell level, SMCs aid in supporting the surrounding ECM by generating contractile forces, or I-O forces, and resisting blood pressure expansion forces, or O-I forces (Chiu and Chien, 2011; Pasta et al., 2013; Pichamuthu et al., 2013). Using our previously reported non-electrospinning Spinneret-based Tunable Engineered Parameters (STEP) technique (Nain et al., 2008, 2009; Nain and Wang, 2013; Wang and Nain, 2014), we have recently developed Nanonet Force Microscopy (NFM, Fig. 1) to measure both I-O and O-I forces at single cell resolution (Sharma et al., 2014; Sheets et al., 2016). In this work, we apply the NFM platform to measure and compare both I-O and O-I forces of SMCs (Fig. 2, Supplementary movies 1 and 2) from three healthy human patient samples to evaluate baseline cell health. Furthermore, since reactive oxygen species (ROS) have been implicated in aneurysmal disease (Phillippi et al., 2009b; Folkersen et al., 2011; Branchetti et al., 2013) and potentially affect a cell’s ability to produce and withstand forces, we determined the influence of ROS exposure on the SMC adhesive forces, thus, providing further insight into the relationship between SMCs and ECM and how disease mechanisms could be studied using this platform. Measurement of human patient single cell forces attached to ECM- mimicking fibers provides new means to calibrate cell forces across multiple patients, thus allowing the framework to study diseased states and response of drugs.

RESULTS
I-O forces during migration and contractile state of SMC adhesion strength

Fused-fiber nanonets were fabricated using non-electrospinning STEP technique. Due to absence of electric source in the fiber spinning process, STEP enables precise control on fiber diameter, spacing and orientation (Nain and Wang, 2013; Wang and Nain, 2014). Using, STEP we developed nanonets at ~15-20 µm spacing, to which cells attached in parallel morphologies with focal adhesions clustered predominantly at the poles (Sheets et al., 2013). Cells utilize their focal adhesions to exert force on their substrate to spread or migrate. To calculate the forces exerted by cells on fibers, the adhesion cluster lengths are modelled as point loads (Sheets et al., 2016). We observed that cells stretched between parallel fibers migrated by forming protrusions synchronously or asynchronously in an oscillatory manner on either fiber (Fig. 3, Supplementary Movie 3). This behavior was maintained amongst all three patient samples, thus suggesting an overall conserved
behavior in cell migration on fibers. Next, to determine the contractile state of the cell, we averaged the total force over one hour for each cell and then averaged the forces for at least 11 cells per patient sample group. The average forces of the SMCs were found to be similar $12.4 \pm 2.4$, $14.5 \pm 1.4$, and $12.9 \pm 0.8$ nN ($p=0.30$) (Fig. 3C). Thus, the average I-O force ($12.9 \pm 1.0$ nN) for the three cell populations established the baseline contractile force for SMCs.

**O-I force provides SMC-fiber adhesion strength**

Using the same parallel-cell morphology, O-I force is measured by uniformly stretching the cell using custom dual-probes positioned on either side of the cell. The probes were moved at a constant stretch rate of 2 µm/sec, thus creating an active and passive fiber system (Fig. 2B, Supplementary Movie 2). To measure the cell-fiber adhesion strength, cells were stretched until they detached from either of the two fibers. By using the two-point load model for the deflection of the passive fiber, we were thus able to calculate the maximum adhesion (O-I) force at detachment. A representative force-time plot in O-I perturbation displays a rise in the force, while adhesion integrity is maintained followed by a sharp decrease indicating cell-fiber adhesion failure (Fig. 4A). O-I forces were calculated for the three cell lines with sample sizes ≥7 cells per population to evaluate consistency across patients and to develop a baseline SMC-fiber adhesion strength metric (Fig. 4B). The mean O-I forces of the three populations were not statistically different ($p = 0.32$). Therefore, we then combined the O-I forces from the three populations to find an average maximum SMC O-I force of $57.9 \pm 2.5$ nN. Although the O-I forces were similar, during the experiments we visually observed variations in cell sizes and that larger cells tended to produce higher forces at failure. We performed linear least-squares regression analysis and found cell area and force at failure to be moderately correlated with an $R^2 = 0.494$ (Fig. 4C) across all populations. This suggests that the size of individual cells may be related to the maximum O-I adhesion force. Combined together, we found an approximately five-fold increase in the cell-fiber O-I compared to the passive contractile I-O forces ($p < 0.01$) (Fig. 4D).

**Effects of oxidative stress and cyclic mechanical stress on adhesion force**

To explore the influence of oxidative stress conditions in our force measurement platform, we exposed the SMCs to a pre-treatment with hydrogen peroxide in the culture medium (oxidative stress conditions). The presence of hydrogen peroxide decreased both the single-cell I-O and O-I forces of human SMCs (Fig. 5). The calculated I-O force in cells exposed to hydrogen peroxide was decreased by about one-half that of SMCs under normal culture conditions ($5.6 \pm 0.7$ vs. $12.9 \pm 1.0$ nN, $p< 0.01$). We also observed a marked increase in the migration rates of cells under oxidative conditions ($168.3\pm18.1$ vs. $70.7\pm10.4$ µm/h, $p=0.011$, inset, Fig. 5A, Supplementary Movie 4). The effect of peroxide on O-I adhesion force was similar to that determined for I-O forces ($57.9\pm 2.5$ nN, $p=0.011$) (Fig. 5B).

Next, we investigated the role of cyclic perturbation on passive contractile and cell-fiber adhesion strength modulation (Supplementary Movie 5). The cells were exposed to a cyclic, sub-failure stretch for 1, 5, 10, and 20 min intervals before a final O-I pull to failure. For all tested cyclic time intervals, we observed a decrease in both the passive contractile force, and the O-I force ($27.8 \pm 3.9$ vs. $57.9 \pm 2.5$ nN, $p<0.01$) (Fig. 5 C-F). The reduction in I-O forces was consistent with our previous observation on reduction in forces with cyclic perturbations (Sheets et al., 2016). I-O forces were reduced by similar amounts at 1 and 5 min cycles ($6.8 \pm 2.6$ and $10.4 \pm 2.8$ %, $p=0.378$) but decreased significantly over long cycles ($24.8 \pm 4.2$ and $64.8 \pm 0.7$ % for 10 and 20 min, respectively, $p<0.02$). However, interestingly we observed the decrease in O-I forces to
occur even at short intervals. As cells form focal adhesions at their poles on fibers, our data suggests that cyclic perturbations cause an active rearrangement of adhesions early on, thus leading to reduced cell-fiber adhesion forces even at short cyclic times.

4 DISCUSSION

The platform presented here, to the best of our knowledge, is the first to measure human SMC single cell-fiber forces in both passive and perturbed states. With our nanonet force platform, we have established a baseline SMC contractile force of 12.9 ± 1.0 nN across populations isolated from multiple patients. In the arterial system, particularly the aorta, tissue must also be able to withstand high O-I stretch forces that results from pulsatile contractions. By perturbing the cells, we have established a reference force of 57.9 ± 2.5 nN for healthy, non-aneurysmal SMCs. These passive and perturbed force calibrations could provide new insights in understanding, diagnosing, and developing preventive strategies for patients with cardiovascular disease.

SMCs bind to and interact with elastin and collagen fibers of the vascular ECM by exerting I-O contractile forces through actin-myosin contractions. The cells’ ability to maintain these forces is necessary to preserve structural integrity and homeostasis of the tissue. For example, specimens of human aneurysm were shown to exhibit a loss of structural integrity as demonstrated by decreased delamination strength of ascending aortic tissue, particularly for the aortopathy associated with bicuspid aortic valve (BAV). (Nataatmadja et al., 2003) Specimens from BAV-related aneurysm also exhibit a unique anisotropic ECM microarchitecture (Phillippi et al., 2014), consistent with marked increases in un axial tensile strength when compared with normal healthy specimens (Pichamuthu et al., 2013). Thus, there is a critical unmet need to develop enabling technologies, which are able to recapitulate contextual in vivo environments, while simultaneously affording access to cells for manipulation and study of putative disease pathways. Such new knowledge on cell-matrix interactions could have a major impact on public health as 1-2% of the United States general population have BAV (Ward, 2000; Gleason, 2005) which carries a heightened risk of developing an aneurysm, and is likely incited by different effector molecules than those causing degenerative aneurysm. In turn, presence of aneurysm places the patient at risk for aortic catastrophe in form of dissection and rupture requiring immediate surgical intervention.

In pathological conditions, SMCs can be exposed to environmental stress (e.g. oxidative stress, ROS, etc.); thus, it is important to understand how I-O and O-I forces change in response under such conditions. In addition to the intracellular response, in this study we are able to show that exposure to oxidative stress imparts a reduction of both I-O and O-I forces by about one-half that of cells under normal culture conditions. When we exposed the non-aneurysmal SMCs to cyclic loading, we observed that transient cell forces (both I-O and O-I) decreased over longer time intervals as cell stretch increased. Interestingly, we found that cyclic loads and oxidative stress reduced the O-I forces to a similar degree. These current findings in primary SMC and the enabling techniques support and enable our ongoing investigations related to SMC force mechanisms in the setting of BAV-associated aortopathy.

Oxidative stress has been of increasing interest in cardiovascular disease, with reactive oxygen species (ROS) linked to hypertension, atherosclerosis and atrial fibrillation (Sahoo et al., 2016). Our prior observations of human aortic specimens noted that medial SMCs isolated from specimens of BAV-associated aneurysm exhibited more oxidative stress-induced cell death when compared with normal aortic specimens and specimens of degenerative aneurysms (Phillippi et al., 2009a, 2010). Furthermore, primary SMCs isolated from BAV patients also exhibited down-regulation of the antioxidant metallothionein when compared with normal aortic SMCs therefore implicating a role for oxidative stress response in BAV-associated aneurysmal disease. In addition, Branchetti and colleagues discovered that increased wall stress results in ROS accumulation, causing
vascular smooth muscle cells to change towards a synthetic phenotype (Branchetti et al., 2013). The force measurement method described here for primary human SMC will help us to further understand how SMC vulnerability to ROS in BAV-associated aortopathy influences the cell-matrix biomechanics that contribute to biomechanical integrity of the aortic wall. Additionally, our nanonet approach to measuring SMC force generation will allow for testing the effect of modulating various cellular and matrix related mechanisms toward our long-term goal of preventing or abating aneurysmal formation.

In conclusion, our studies emphasize that a combinatorial strategy of measuring both I-O and O-I forces should be implemented to develop a comprehensive understanding of the role of forces in cardiovascular disease. With our contextually relevant nanonet force platform, we have demonstrated that oxidative stress conditions resulted in perturbed adhesion forces in healthy non-aneurysmal SMCs in complex in vitro structures that mimic the ECM milieu. The capability to alter the in vitro fibrous dimensions and measure corresponding force generation in human SMCs will be invaluable towards understanding aortic wall integrity via cell-matrix adhesion. To our knowledge, this approach has never been applied to understand mechanisms of aortic aneurysmal disease. In the future, we plan to incorporate this platform to expand our studies of cell-cell and cell-matrix interactions in aortic disease pathologies at the cell-cell junctions and focal adhesion levels respectively, to aid in development of therapeutic strategies designed to prevent progression of aneurysm and rupture of aorta.

MATERIALS AND METHODS

Non-electrospinning STEP fused-fiber nanonets: The previously reported non-electrospinning STEP technique was used to fabricate suspended crisscross polystyrene nanonet scaffolds with fused fiber interactions (Fig. 1). Previous work from our group has demonstrated that cells on suspended nanofibers tend to spread between two parallel fibers if the gap between them is <20µm (Meehan and Nain, 2014). To increase the occurrence of this cell shape, the nanonets were configured such that strut-like, ~1000 nm-diameter fibers, spaced ~300 µm apart, were spun orthogonal to and on top of smaller ~250 nm-diameter fibers with tighter spacing (15-20 µm apart).

Aortic tissue collection and SMC culture:
Human ascending aortic specimens were collected from patients presenting for surgery to the Division of Cardiac Surgery, University of Pittsburgh Medical Center for heart transplantation and were collected under IRB-approval and with informed patient consent or from organ donors via the Center for Organ Recovery and Education. Aortic specimens were collected from one male and two female patients ranging in age from 41 to 62 yr. Primary medial SMCs were isolated as previously described (Phillippi et al., 2009a). Three non-aneurysmal cryopreserved SMC cell populations were re-established in culture at passages 3-6 in 25 cm² flasks. The STEP scaffolds were fixated to the culture well of a glass-bottom 6-well plate and glass-bottom dishes using sterile vacuum grease for I-O and O-I experiments, respectively. The fibers were sterilized by adding 1 mL 70% ethanol for 10 minutes followed by washing with PBS. The fibers were then coated in 4 µg/mL fibronectin (Invitrogen, Carlsbad, CA) for 1-2 hours to assist with cellular attachment onto the fibers (Sheets et al., 2013). At 80% confluence, the cells were trypsinized and diluted in SMC medium (Cat#: 311K-500, Cell Applications Inc.) to a concentration of 30x10⁴ cells/mL and then seeded on the scaffolds by placing 35 µL droplets on top of the fiber grids. The cells were allowed to attach to fibers in an incubator at 37°C and 5% CO₂ for 4-6 hours. Afterward, 2 mL of SMC media was added to immerse the scaffold. For inducing oxidative stress, the cells were exposed to 125 µM hydrogen peroxide (Sigma-Aldrich), freshly-diluted SMC medium for 30min (Lennon et al., 1991).

Imaging:
For I-O and O-I measurements, after the cells were allowed to attach to the STEP fibers and 2 mL of SMC media had been added to the 6-well plate, the plate was placed into an incubating microscope with a digital three-axis stage (AxioObserver Z1; Carl Zeiss, Germany). Cells are visualized using 20x magnification with an AxioCam MRm camera (Carl Zeiss). In I-O experiments, time-lapse was performed with images captured at 1 minute intervals and care was taken to image cells in the parallel shape and avoid imaging cells in contact with other cells.

O-I forces were measured by placing glass micropipette probes mounted on a MP-285 motorized manipulator (Sutter) on either side of a parallel cell. To capture O-I force measurements, time-lapse images were taken every 600 ms. As with I-O forces, images were taken under 20x magnification. In oxidative stress condition experiments, cells were imaged for 15-20 min before adding the H$_2$O$_2$ media to visualize transient force modulation. Force data was analyzed using a custom MATLAB code and the software ImageJ (National Institutes of Health, Bethesda, MD).

**Force Measurement:**

As cells attach and spread between parallel nanonet segments, the individual fibers deflect (Fig 2, Supplementary Movie 1). In this configuration, cells form cell-fiber adhesion clusters on the periphery on each fiber (Meehan and Nain, 2014). Due to these clusters, we assume that a cell applies two point loads on the fiber, one on each end of the cell-fiber interface (where the focal adhesions cluster) which both contribute to overall fiber deflection (Sheets et al., 2016). Thus, the experimentally measured deflections $\delta_a$ and $\delta_b$ (Fig. 2B) can be related to their associated loads $P_a$ and $P_b$ (Sheets et al., 2016):

$$\delta_a = \left( \frac{P_a \sinh(\lambda(L - a))}{S \sinh(\lambda L)} - \frac{P_b \sinh(\lambda(L - b))}{S \sinh(\lambda L)} \right) \alpha + \left( \frac{P_a \sinh(\lambda(L - a))}{S \sinh(\lambda L)} + \frac{P_b \sinh(\lambda(L - b))}{S \sinh(\lambda L)} \right) \frac{\sinh(\lambda a)}{\sinh(\lambda L)}$$

$$\delta_b = \left( \frac{P_a \sinh(\lambda(L - a))}{S \sinh(\lambda L)} + \frac{P_b \sinh(\lambda(L - b))}{S \sinh(\lambda L)} \right) \alpha + \left( \frac{P_a \sinh(\lambda(L - a))}{S \sinh(\lambda L)} - \frac{P_b \sinh(\lambda(L - b))}{S \sinh(\lambda L)} \right) \frac{\sinh(\lambda b)}{\sinh(\lambda L)}$$

with the dependent variables defined in Table 1.

As cells migrate, they apply contractile forces on outer fibers, causing them to deflect (Fig. 2A), from which I-O forces can be calculated. In the present study, we only considered cells attached to a 2-fiber system. To develop the overall contractile force of the cell at any instant, we summed the individual fiber forces. To compare forces across different cells within and across SMC populations, the I-O contractile forces were collected and averaged over 1 hour of imaging. We compared these forces for three different human patients with a sample size of $\geq 11$ cells per population.

O-I force is measured from cells in the same parallel morphology by uniformly pulling one of the fibers with a force probe until cell detachment, thus creating an active and passive fiber system (Fig. 2B, Supplementary Movie 2). The deflection of the passive fiber is used to calculate force, and the point of cellular detachment from the passive fiber is considered the maximum O-I adhesion force of the cell to the fiber. The probes were fixed with their tips ~200$\mu$m apart to account for larger cell widths and at a 45-degree angle to prevent the fiber slipping vertically during pulling. Using the manipulator, the probes were positioned parallel to and evenly spaced about the cell, thus providing uniform stretch to the cell in single and cyclic force perturbation modes. For cyclic mode, cells were cyclically stretched by programming 1, 5, 10 and 20 min intervals at a 2$\mu$m/sec strain-rate before being pulled to adhesion failure. The cells were stretched to induce an initial 20 nN force, resulting in a 3 cycle/min frequency.

**Statistical Analysis:** I-O and O-I forces of the three populations were tested for statistical significance with one-way ANOVA tests for difference of means to a p-value $\leq 0.05$ (unless otherwise noted). The same test and p-value were used to evaluate the effects of
oxidative stress and cyclic loading to unstressed and non-loaded populations. Linear least-squares regression was also used to evaluate trends in cell-area vs. O-I force. Error bars represent standard error unless otherwise noted.

ACKNOWLEDGEMENTS

TG and JP would like to acknowledge the support provided by the National Heart, Lung and Blood Institute of the National Institutes of Health (NIH) under Award Number HL 109132 (TGG) and the Department of Cardiothoracic Surgery at the University of Pittsburgh. TG and JP gratefully acknowledge Kristin Konopka and Julie Schreiber for assistance with IRB protocols and informed patent consent, Jennifer Hill and Tara Richards for smooth muscle cell isolation, and the Center for Organ Recovery and Education for assistance in obtaining donor tissues. ASN would like to acknowledge the support provided by National Science Foundation (NSF) grant CMMI-1437101 and 1462916 and the Institute for Critical Technology and Applied Sciences (ICTAS) at Virginia Tech.

REFERENCES


Figure Legends

**Figure 1:** Fused-fiber nanonets. Scanning electron microscope (SEM) images of nanonets spun over a hollowed-out scaffold such that large-diameter (~1000 nm), widely spaced fibers are orthogonal to and overlay small-diameter (250 nm), closely spaced fibers. Both fiber layers are fused at the intersections as shown by the red dashed oval. In such a configuration, cells exclusively interact with suspended fibers.
Figure 2. Optical image of SMCs attached to nanonets. (A) Inside-out (I-O) force measurement in parallel shapes on 2 and 3-fiber groups, and (B) Outside-in (O-I) of single cell stretched by two probes on either side shown by black circles. Red arrows show representative fused-fiber intersections. Experimental measurements are shown only for O-I case, with dashed white line representing undeflected position of fiber and $\delta_a$, $\delta_b$, $a$, and $b$ represent the physical measurements made to estimate cell forces.

Figure 3. (A) Optical time lapse images showing oscillatory pattern of protrusions on parallel fibers during cell migration. Time shown in hours:minutes:seconds:thousandths, (B) Forces of top and bottom protrusions at the leading edge, and (C) Average inside-out force values amongst three human patient samples. Statistically, these values were not significantly different ($p=0.30$) and error bars represent standard error.
Figure 4. (A) Typical transient force response curve for cell undergoing O-I perturbation with a drop at 60 second point indicating the maximum cell-fiber adhesion strength, (B) The average O-I forces for the three populations with no significant variation, (C) Linear least-squares regression analysis shows failure forces and area at failure to be moderately correlated across all SMC populations undergoing O-I perturbation ($R^2 = 0.494$), and (D) I-O and O-I force comparison with force values averaged across the cell populations, showing a five-fold difference in the I-O and O-I force ($p<0.01$). Error bars represent standard error.
Figure 5. (A, B) Effect of oxidative stress on force modulation of SMCs for both I-O and O-I modes (p<0.01 for both cases). Inset in (A) shows the higher migration rate of cells under oxidative conditions (p<0.01). (C, D) Representative transient profiles of cyclic perturbation showing decrease in force with increasing number of cycles and the detachment of cell from fiber in O-I mode (shown by arrow). (E) I-O forces decrease with increasing number of cycles, and (F) O-I forces decrease with cyclic perturbation.

Tables

Table 1. STEP Nanonet force microscopy model parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Significance</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>L</td>
<td>Segmental length of the fiber (distance between adjacent fixed ends)</td>
<td>~300 (µm)</td>
</tr>
<tr>
<td>a</td>
<td>Location of point load nearest to fiber-fiber intersection</td>
<td>0 &lt; a &lt; L (µm)</td>
</tr>
<tr>
<td>b</td>
<td>Location of other point load</td>
<td>0 &lt; a &lt; b &lt; L (µm)</td>
</tr>
<tr>
<td>δa</td>
<td>Fiber deflection at a</td>
<td>Variable (µm)</td>
</tr>
<tr>
<td>δb</td>
<td>Fiber deflection at b</td>
<td>Variable (µm)</td>
</tr>
<tr>
<td>λ</td>
<td>Shape-dependent mechanics parameter</td>
<td>√ S / EI (m⁻¹)</td>
</tr>
<tr>
<td>S</td>
<td>Uniform pre-tensional load</td>
<td>S = π(d²/4) * T (µN)</td>
</tr>
<tr>
<td>T</td>
<td>Uniform pre-tensional stress, calculated from AFM residual stress measurements</td>
<td>4.1 MPa(Wang, 2015)</td>
</tr>
<tr>
<td>d</td>
<td>Fiber diameter</td>
<td>~250 nm</td>
</tr>
<tr>
<td>E</td>
<td>Elastic modulus of the polymer (polystyrene)</td>
<td>E = 0.97 (GPa)(Wang, 2015)</td>
</tr>
<tr>
<td>I</td>
<td>Area moment of inertia</td>
<td>I = πd⁴ / 64 (m⁴)</td>
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