Histological evaluation of flexible neural implants; flexibility limit for reducing the tissue response?

Heui Chang Lee1,2,7, Fredrik Ejerholm3,4,7, Janak Gaire5, Seth Currlin5, Jens Schouenborg1, Lars Wallman3,4, Martin Bengtsson3,4,8, Kinam Park2,6 and Kevin J Otto2,5,9

1 Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN, United States of America
2 J Crayton Pruitt Family Department of Biomedical Engineering, University of Florida, Gainesville, FL, United States of America
3 Department of Biomedical Engineering, Lund University, Lund, Sweden
4 NeuroNano Research Centre, Lund University, Lund, Sweden
5 Department of Neuroscience, University of Florida, Gainesville, FL, United States of America
6 Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN, United States of America

E-mail: kevin.otto@bme.ufl.edu and Martin.Bengtsson@bme.lth.se

Received 30 October 2016, revised 22 March 2017
Accepted for publication 24 March 2017
Published 4 May 2017

Abstract

Objective. Flexible neural probes are hypothesized to reduce the chronic foreign body response (FBR) mainly by reducing the strain–stress caused by an interplay between the tethered probe and the brain’s micromotion. However, a large discrepancy of Young’s modulus still exists (3–6 orders of magnitude) between the flexible probes and the brain tissue. This raises the question of whether we need to bridge this gap; would increasing the probe flexibility proportionally reduce the FBR? Approach. Using novel off-stoichiometry thiol-enes-epoxy (OSTE+) polymer probes developed in our previous work, we quantitatively evaluated the FBR to four types of probes with different softness: silicon (~150 GPa), polyimide (1.5 GPa), OSTE+Hard (300 MPa), and OSTE+Soft (6 MPa). Main results. We observed a significant reduction in the fluorescence intensity of biomarkers for activated microglia/macrophages and blood-brain barrier (BBB) leakiness around the three soft polymer probes compared to the silicon probe, both at 4 weeks and 8 weeks post-implantation. However, we did not observe any consistent differences in the biomarkers among the polymer probes. Significance. The results suggest that the mechanical compliance of neural probes can mediate the degree of FBR, but its impact diminishes after a hypothetical threshold level. This infers that resolving the mechanical mismatch alone has a limited effect on improving the lifetime of neural implants.

Keywords: flexible brain-computer interface (fBCI), off-stoichiometry thiol-enes (OSTE), chronic neural implant, foreign body response (FBR), neuroprosthetics

(Some figures may appear in colour only in the online journal)
1. Introduction

One primary objective of the neuroprosthetic field has been to engineer devices that are capable of interfacing with tissue for dozens of years in order for neural implants to be considered a viable treatment option for individuals with neurological disorders [1, 2]. The functionality of implanted microelectrodes typically degrades over time and is reported to be associated with a severe foreign body response (FBR) that persists as the implant stays in the brain [3, 4]. A number of strategies have been proposed to improve the brain’s FBR to neural implants via modulating mechanical and/or biochemical aspects of the device-tissue interface [2]. In order to integrate multiple approaches to devise a complex, multimodal solution, identifying individual factors that critically impact the FBR has been of particular importance [6].

The development of micro-fabricated neural probes as an intracortical interface has come a long way from Wise’s approach in the 1970’s [7]. While the flat sword shape design remains unchanged, the development of new materials has opened up possibilities for improved functionality. Within the device design approach [55], the mechanical mismatch of the soft brain tissue and the rigid neural implant has been thought to be one of the key components that needs mediation [8]. Soft brain tissue and the rigid neural implant has been thought to be one of the key components that needs mediation [8].

While the flat sword shape design remains unchanged, the development of new materials has opened up possibilities for improved functionality. Within the device design approach [55], the mechanical mismatch of the soft brain tissue and the rigid neural implant has been thought to be one of the key components that needs mediation [8]. The flat sword shape design remains unchanged, the development of new materials has opened up possibilities for improved functionality. Within the device design approach [55], the mechanical mismatch of the soft brain tissue and the rigid neural implant has been thought to be one of the key components that needs mediation [8]. The flat sword shape design remains unchanged, the development of new materials has opened up possibilities for improved functionality. Within the device design approach [55], the mechanical mismatch of the soft brain tissue and the rigid neural implant has been thought to be one of the key components that needs mediation [8].

The key finding was that flexible probes decrease both tangential and radial strains at any given degree of device tetheredness or device-tissue integration, which was further evaluated in vivo by Sridharan et al. [14]. In vitro cell viability studies pertaining to mechanotransduction also suggested that excessive strain conditions decrease neuronal viability [15], whereas soft substrates allow for preferential attachment and growth of neurons over glial cells [16]. Based on these initial findings, many groups shifted gears toward developing probes made of softer materials than silicon, including polyimide [8, 17], parylene [18, 19], SU-8 [20, 21], nanocomposites [14, 22–24], and other materials [25–29].

Although flexible probes were found to induce significantly less strain–stress, the impact of reduced strain–stress to both the in vivo FBR and the device performance needed to be verified. Quantitative histological studies by the Capadona group and the Tyler group demonstrated that nanocomposite probes, which becomes extremely flexible (i.e. 12 MPa) when hydrated, induce significantly less neuroinflammatory response than standard silicon probes and polyvinyl acetate (PVAc)-coated silicon probes [23, 24]. In addition, several other studies showcased enhanced long-term electrophysiological characteristics in the central nervous system (CNS) with flexible surficial [30] or penetrating devices [20, 31].

It was becoming more evident that flexible materials are a favorable choice for neural implant substrates. However, questions remained regarding whether the increase in flexibility reduces the FBR proportionally, or only up to a certain level. Analogous to the critical surface area hypothesis by Seymour et al. [32], there may be a critical flexibility level, beyond which the performance benefit would plateau.

In order to evaluate probes that are softer than polyimide we used probes made of off-stoichiometry thiol-enes-epoxy (OSTE+) polymers, which is synthesized by a radical-mediated thiol-ene click reaction. OSTE+ polymer was initially developed for microfluidic devices [33] and advanced to neural implant application [26]. The strength of the OSTE+ is its patternable nature (due to the use of UV-lithography) and tunable Young’s modulus (by simply changing the ratio of constituents). With this feature we created two types of OSTE+ probes made of the same materials that are moderately soft (OSTE+Hard) and very soft (OSTE+Soft), to solely evaluate the effect of flexibility. Our previous in vitro study suggested that the unbound constituents of OSTE+ were effectively removed by incubating the probe in water for 7 d [34]. The results of this study suggest that comparing conventional probes with OSTE+ probes would reveal the consequence of flexibility on the severity of the tissue response.

We evaluated the FBR of four microelectrodes with differing flexibility: silicon, polyimide, OSTE+Hard, and OSTE+Soft. Young’s moduli of the three polymer probes were obtained with dynamic mechanical thermal analysis. Then, an in vivo study was conducted to quantitatively compare the FBR of the four types of probes at 4 and 8 weeks post-implantation (WPI). The CX3CR1-GFP mouse strain [35], widely utilized in neuroscience research [36], was used to visualize microglia with endogenous green fluorescence. In order to provide the structural rigidity for insertion into mice pia mater, 4 kDa polyethylene glycol (PEG) was dip-coated onto probes.

2. Materials and methods

Chemicals were purchased from Sigma Aldrich (USA) unless otherwise specified.

2.1. Fabrication of probes

2.1.1. OSTE+ probes. As described in previous work [26, 34], OSTE+ is polymerized in two steps: 1) a fast UV initiated radical polymerization between a thiol and two allyls and 2) a slower thermal anionic polymerization between a residual thiol and an epoxy resin. The first polymerization utilizes Irgacure® TPO-L (BASF, Germany), an UV-initiator that crosslinks the thiol [tris(2,3-mercapropropionlyxethyl) isocyanurate (BRUNO BOCK, Chemische Fabrik GmbH and Co. KG, Germany) with the two allyls, trimethylolpropane diallyl ether and 2,4,6-trialylxyloxy-1,3,5-triazine. The second polymerization utilizes DBN (1,5-diazabicyclo[4.3.0]non-5ene) as the initiator that crosslinks the unreacted thiol and the epoxy resin (D.E.N 341 Epoxy Novolac resin, Dow Chemicals, USA).
Table 1. Fabrication parameters for OSTE\textsuperscript{+}\textsubscript{Hard} and OSTE\textsuperscript{+}\textsubscript{Soft}.

<table>
<thead>
<tr>
<th>Blend</th>
<th>% more thiol than allyl</th>
<th>Stoichiometric mixing ratio</th>
<th>Spin speed</th>
<th>Spin time</th>
<th>UV exposure time</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSTE\textsuperscript{+}\textsubscript{Hard}</td>
<td>65%</td>
<td>1.65 : (0.05 : 0.95) : 0.65</td>
<td>2000–3000 rpm</td>
<td>30 s</td>
<td>75 s</td>
</tr>
<tr>
<td>OSTE\textsuperscript{+}\textsubscript{Soft}</td>
<td>20%</td>
<td>1.20 : (0.95 : 0.05) : 0.20</td>
<td>500–1000 rpm</td>
<td>30–150 s</td>
<td>60 s</td>
</tr>
</tbody>
</table>

Thiols, allyls, and epoxy were mixed according to table 1, with 0.2 wt% of DBN and TPO-L. Triallyl and epoxy were preheated to 67 °C before mixing. After degassing, the mixture was spin coated onto a silicon wafer having a 200 nm sacrificial layer of titanium according to table 1. Then, a 9 µm thick PVC film was applied to wafers to prevent the mixture from sticking to the UV mask and from reacting with the oxygen. Wafers were exposed to the UV light as listed in table 1 using a MA4 mask aligner (Carl Suss, Germany). Then, wafers were submerged in butyl acetate for 10–15 s to remove the PVC film and rinsed in toluene for 5 s. Wafers were blown dry with nitrogen gas and placed in an oven at 67 °C for 2 d. Prior to removing the sacrificial layer to release the probes, wafers were incubated in MilliQ water at room temperature (RT) for 6 d. Wafers were blown dry and slightly etched using an oxygen plasma etcher (Diener Femto, Germany) for 5 min in order to remove a residual layer of OSTE+ left after the development. Probes were released by incubating the wafers in 8% hydrofluoric acid solution for 24 h. After rinsing with MilliQ water several times the probes were released using a pair of tweezers.

### 2.12. Polyimide probes.

Polyimide (Durimide 7520, FujiFilm, Belgium) was spin coated onto a silicon wafer (200 nm) on a hot plate at 100 °C for 3 min prior to UV-light exposure for 45 s. Wafers were allowed to rest overnight at RT. Then, wafers were developed using HTR-D2 (FujiFilm) for 4 min and rinsed with RER600 (FujiFilm). Wafers were blown dry, hard baked in an oven at 180 °C for 2.5 h, and submerged in MilliQ at RT for 6 d. Probes were released in the same way as the OSTE+ probes using hydrofluoric acid and MilliQ.

### 2.2. Fabrication of samples for dynamic mechanical thermal analysis (DMTA)

For the DMTA testing, samples of each polymer were made in blocks (1 mm thick, 10 mm wide, and 40 mm long). Samples were submerged in MilliQ at RT for 6 d after fabrication.

For OSTE+ block samples, thiols, allyls, and epoxy were mixed and degassed in the same way as the OSTE+ probe fabrication procedure. Then, the degassed mixture was poured onto a PDMS mold, covered with a polycarbonate film, and exposed to the UV-light for 40 min (using a Carl Suss MA4 mask aligner). After the exposure, the polycarbonate film was removed and samples were demolded and hard baked in an oven at 67 °C for 2 d.

For polyimide block samples, polyimide was poured onto a PDMS mold followed by a soft-bake at 67 °C for 2 d. The amount of polyimide was adjusted accordingly to compensate for the shrinkage. The mold was then exposed to UV-light for 40 min (using a Carl Suss MA4 mask aligner) and samples were demolded and hard baked in an oven at 180 °C overnight.

A dynamic mechanical analyzer (Q800, Texas Instruments, USA) was used to test the Young’s moduli of the three polymer block samples. Tension was the mode of deformation. Samples were tested from 5 °C to 45 °C with a 2 °C step size at a frequency of 1 Hz. Three samples of each polymer were tested (N = 3).

### 2.3. In vivo implantation

#### 2.3.1. Probe preparation.

One day before implantation surgery, four types of probes: silicon (GP1x16_249 (NeuroNexus Technologies, USA), 249 µm wide, 15 µm thick, 18° tapered tip), polyimide, OSTE+\textsuperscript{+}\textsubscript{Hard} and OSTE+\textsubscript{Soft} were autoclaved at 121 °C for 30 min. All probes were then dip-coated with sterilized 4 kDa poly-ethylene-glycol in an aseptic setting and stored in a sterilized container until surgery.

#### 2.3.2. Surgical implantation.

All surgical procedures and animal care practices were performed in accordance with the University of Florida Institutional Animal Care and Use Committee (IACUC).

Twenty-two adult mice, 20–24 g, (B6.129P-Cx3cr1/J (Jackson Laboratories, USA), N = 5–6 per condition) were bilaterally implanted with one probe per hemisphere. Mice were anesthetized with isoflurane (Abbott Laboratories, USA, 3% induction, 1.5% maintenance) and mounted onto a stereotactic frame. Mice were kept warm on a heating pad and their vital signs were monitored using a pulse oximeter (MouseOx, Kent Scientific, USA). After a small portion of scalp was removed, a craniotomy was made on each hemisphere centered at 1.5 mm lateral and 1.5 mm posterior to bregma. Probes were inserted 1.2 mm deep using a piezoelectric actuator (PILine M-663, Physik Instruments, Germany) at 100 mm s\(^{-1}\). Identifiable blood vessels were avoided to minimize iatrogenic variability. Then, the upper portion of the probe was trimmed with a pair of microscissors to detach from the actuator. Kwik-Sil (World Precision Instruments, USA) was applied to the craniotomies and tethered the probes followed by dental acrylic (Fusio Liquid Dentin, Pentron, USA) to secure the headcap.

### 2.4. Immunohistological processing

#### 2.4.1. Tissue preparation.

At 4 WPI or 8 WPI, mice were deeply anesthetized with 5% isoflurane and transcardially perfused with 20 ml of cold phosphate buffered saline (PBS), followed by 20 ml of cold 4% paraformaldehyde solution (PFA) [37]. After post-fixing heads for 24 h at 4 °C brains were carefully extracted while retaining the probes in place. Brains were...
Table 2. List of primary antibodies.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Target of interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD68 (1:250, ab53444, Abcam)</td>
<td>Activated microglia/macrophages</td>
</tr>
<tr>
<td>GFP (Autofluorescent)</td>
<td>Microglia/macrophages</td>
</tr>
<tr>
<td>Anti-IgG (1:250, ab150114, Abcam)</td>
<td>Blood-brain barrier leakiness</td>
</tr>
<tr>
<td>Anti-GFAP (1:500, AB5541, Millipore)</td>
<td>Astrocytes</td>
</tr>
<tr>
<td>Anti-NeuN (1:250, MABN140, Millipore)</td>
<td>Neuronal nuclei</td>
</tr>
</tbody>
</table>

* GFP is an endogenously expressed fluorophore in CX3CR1-GFP mice.

cryopreserved in 30% sucrose in PBS at 4 °C for 24–48 h. Then, brains were flash frozen in 2-methyl-butane at −40 °C for 2 min and transferred to a cryostat (CM1950, Leica, Germany). Brains were lightly embedded with optimum cutting temperature (OCT) compound (Sakura Finetek, The Netherlands) and sliced into 25 μm horizontal sections with the retained probes. Tissue slices were collected on SuperFrost glass slides (Thermo Fisher, USA) and stored at 4 °C for the immunostaining process.

2.4.2. Immunostaining. Tissue slices were allowed to sit at RT for 30 min to adhere to the slides. After washing for 5 min in PBS three times, tissue slices were blocked in blocking buffer (4% v/v goat serum, 0.3% Triton-X in PBS) for 2 h at RT. Primary antibodies (refer to Table 2) were diluted in blocking buffer and applied to the slices for incubation at 4 °C for 20–24 h. Following primary incubation, slices were washed for 5 min in PBS five times. Slices were then incubated in secondary antibodies at RT for 2 h. Following secondary incubation, slices were washed for 5 min in PBS five times and coverslipped using mounting medium (H-1000, Vector Lab, USA).

2.4.3. Imaging and quantitative analysis. Fluorescence images were taken with a laser scanning confocal microscope (LSM 710, Carl Zeiss, Germany). A 2 × 2 tile was captured using a 10 × objective that spans the device-tissue interface and its surroundings. For quantitative analysis, two slices per sample, roughly from 400 to 600 μm down the cortical column, were used to minimize the depth-dependent variability [37]. Qualitative figures presented in this paper were contrast enhanced for visual clarification. For better identification of the relationship between immunolabels, representative images are from the same animal hemisphere, horizontal slices within 100 μm to each other.

MINUTE v1.5 was used for fluorescence intensity analysis [24, 38, 49]. An ellipse was drawn to locate the contour of the device-tissue interface. Exclusion areas were drawn to discard areas outside the cortical hemisphere of interest. For every 5 μm concentric ring from the probe contour, average immunolabel intensities were computed. Then a 5-tap moving Gaussian window was applied to smoothen the intensity curves. All average intensities were normalized to the background, taken as the interval 600–800 μm from the probe contour. Neuronal nuclei (NeuN) were automatically counted using a custom built ImageJ [39] script.

Figure 1. Mean Young’s moduli of silicon, polyimide, OSTE+Hard, and OSTE+Soft (A) as a function of the temperature and (B) at 20 °C and 37 °C (with ± standard deviation). The three polymer samples were measured using a dynamic mechanical analyzer.

Statistical analyses were performed using Prism 7.00 statistical analysis software (GraphPad Software, USA). Integrated immunolabel intensities were binned into 50 μm intervals from 0 μm to 500 μm and averaged by each area. A two-way ANOVA was performed with post-hoc Tukey’s test with probe type and distance from the probe as the two independent variables.

3. Results

3.1. Probe specification

The thickness of the polymer probes was measured using a microscope (Olympus BX40, Sweden) with a height sensor. Measured thicknesses were: 21.3 ± 1.0 μm (polyimide), 23.5 ± 2.1 μm (OSTE+Hard), and 22.4 ± 2.1 μm (OSTE+Soft) (mean ± standard deviation, N = 15). Probes were 250 μm wide and 3 mm long with a tapered tip of approximately 18° in angle.

The thickness of the dip-coated PEG on the silicon probe was measured using a profilometer (Alpha Step 500, Tencor, USA). The mean thickness of the thickest portion was 46.05 ± 14.58 μm (mean ± standard deviation, N = 9) on one side of the broad surface.

3.2. Dynamic mechanical thermal analysis (DMTA)

Figure 1 shows mechanical properties of the three polymers compared to silicon at given temperatures. Polyimide (1.5 GPa) was roughly three orders of magnitude softer than silicon (~150 GPa), approximately 5 times harder than OSTE+Hard (300 MPa) and approximately 250 times harder
than OSTE+ soft (6 MPa) at 37 °C. All of the three polymers showed a decrease in Young’s modulus with an increase in temperature. OSTE+Hard showed the largest decrease in Young’s modulus in the tested temperature range. OSTE+Hard was softer than polyimide at 37 °C but harder at 20 °C. We see that the change in Young’s modulus of OSTE+ samples according to the temperature were linear-quadratic in the log-arithmic scale.

3.3. Activated microglia/macrophages (CD68)

CD68 is a glycoprotein present in monocyte derived immune cells, and is most intensely expressed in their activated state [40]. Figure 2 shows that, at 4 WPI, silicon probes had significantly greater CD68 fluorescence intensity than the three polymer probes in the 0–50 µm bin with the most dramatic difference within the first 20 µm. At 8 WPI, silicon probes had significantly greater CD68 than polyimide and OSTE+Hard probes in the 0–50 µm bin. Unlike 4 WPI, at 8 WPI the mean intensity of CD68 around silicon probes was more dispersed and not as concentrated in the first 20 µm. The mean intensity generally decreased from 4 WPI to 8 WPI for all types of probes, with the greatest decrease seen with silicon. The decrease was most pronounced in the 0–50 µm bin whereas regions beyond 50 µm stayed relatively consistent.

3.4. All microglia/macrophages (GFP)

GFP is expressed in monocyte derived immune cells of CX3CR1-GFP transgenic mice, labeling both activated and non-activated microglia/macrophages in the brain [35]. In the 0–50 µm bin, silicon probes had significantly greater GFP intensity than polyimide probes at 4 WPI, as well as all three polymer probes at 8 WPI. GFP expression near all the probes was more amorphously aggregated than the outer region where resting microglia are detected. By 100 µm there was no noticeable difference in the distribution of microglia compared to regions beyond 500 µm. The mean GFP fluorescence intensity generally decreased from 4 WPI to 8 WPI. Unlike CD68, however, the decrease was minimal. CD68 was mostly co-localized with GFP, but not vice versa.

3.5. BBB leakiness (IgG)

Immunoglobulin G (IgG) is the most abundant antibody in blood serum and is often used in chronic inflammatory
studies to indicate leakiness of the BBB in the brain parenchyma [42]. In the 0–50 µm bin, silicon probes had significantly greater IgG fluorescence intensity than OSTE+Hard probes at 4 WPI, as well as polyimide and OSTE+Hard probes at 8 WPI. OSTE+Soft probes had significantly greater IgG than polyimide and OSTE+Hard probes at 8 WPI in the 0–50 µm bin. Although the mean IgG intensity of silicon probes was greater than OSTE+Soft probes at 8 WPI, no statistical difference was found between the two types of probes. Large inter-animal variabilities were observed within the same type probes, manifesting high standard errors. This variability was especially notable around silicon probes at 8 WPI up to 100 µm from the implant. The trend of IgG generally corresponded with CD68 and GFP in terms of silicon probes exhibiting the highest FBR than the other probes. Unlike CD68, there was no notable change in the mean intensity at 8 WPI compared to 4 WPI.

3.6. Astrocytes (GFAP)

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is expressed in mature astrocytes, and more intensely expressed in reactive astrocytes [41]. At 4 WPI, polyimide probes had significantly greater GFAP fluorescence intensity than OSTE+Hard probes in the 0–50 µm bin. At 8 WPI, silicon probes had significantly greater GFAP intensity than OSTE+Soft probes in the 0–50 µm bin. Mean GFAP intensity profile across probes types became more similar to each other at 8 WPI than at 4 WPI. At both times, GFAP intensity mostly dropped down to the background level after 150 µm.

3.7. NeuN density

NeuN were identified with the NeuN immunolabel. Although significant differences were found with other inflammatory markers, no statistical significance was found between any two types of probes at 4 WPI and 8 WPI. Mean neuronal densities approached background levels from approximately 100 µm away from the implant. Mean neuronal densities of silicon probe and polyimide probe were higher at 8 WPI than at 4 WPI in the 0–50 µm bin, although the differences were statistically not significant. Mean background neuronal densities were 2894 ± 290 cells mm⁻² (4 WPI) and 2493 ± 381 cells mm⁻² (8 WPI) (mean ± standard deviation, N = 11).
4. Discussion

Our work herein supports the notion that flexible probes are a favorable choice for neural implants. There was a significant reduction in the mean intensities of FBR markers surrounding the three polymer probes compared to silicon probes. However, it appears that further increasing the flexibility beyond polyimide failed to attenuate the FBR. The difference in Young’s modulus between silicon and polyimide, the stiffest of the three polymers, was roughly 100 times. Although the difference in Young’s modulus between the polyimide probe and OSTE+Soft was roughly 250 times, we did not find any consistent statistical differences in the inflammatory markers between the pairs of polyimide, OSTE+Hard, and OSTE+Soft probes. From this, we infer that flexibility plays a critical role when the probe is relatively hard, but its effect diminishes as the probe gets softer.

The three polymer probes generally showed a reduction in the FBR markers compared to silicon probes. CD68 intensity indicated that there was a significantly more persistent microglial and macrophage activation around silicon probes than the polymer probes. We also found that there was a large reduction in CD68 intensity at 8 WPI than at 4 WPI for all types of probes. This corresponds with previous studies that showed consistent reduction in microglial/macrophage activation after 2 WPI up to 16 WPI [24, 43]. GFP also pointed toward the same conclusion that silicon probes induced the most intense microglial/macrophage response. The overall trend at 4 WPI and 8 WPI both suggest that CD68 and GFP responses were most intense around silicon probes, despite small variations in the type of probes being significantly different versus silicon probes. A region with high CD68 response always accompanied GFP response. However, their relative intensities did not exactly match. It has been reported that a higher-level GFP expression was observed in monocyte-derived macrophages of CD68-GFP mice compared to CX3CR1-GFP mice [44]. It infers a preferential expression of CD68 in myeloid lineage cells and GFP in brain-resident microglia, although GFP is also weakly expressed where CD68 is expressed. With this inherent difference, consistency observed with CD68 and GFP intensity in our study strengthens the statistical significance found in immune cell distribution.

Similar to CD68 and GFP, IgG intensity indicated that silicon probes induced the most severe chronic BBB leakage. However, OSTE+Soft also had significantly higher IgG intensity compared to the other two polymer probes at 8 WPI.
As OSTE+Soft only differed with OSTE+Hard in the constituent mixing ratio and the resulting softness, it is unlikely that OSTE+Soft probes chemically or physically perturb BBB more than OSTE+Hard probes. It is possible that localized IgGs seen only around the probes, within the tight astroglial junction, depends more on the insertion injury and less on the chronic BBB leakage [45]. Note that in general, IgG was particularly variable among the inflammatory markers used in this study. Even among the silicon probes only a few exhibited a very high IgG response near the probe (i.e. 2/5 at 4 WPI and 1/5 at 8 WPI, more than twice greater than the median), rendering the overall mean high. This implies that there may be an important contributor to the FBR other than the extent of the tissue damage, such as the existence or nonexistence of vasculature near the probe. It is speculated that the BBB integrity depends on the chance of mechanical/biochemical damage to nearby large blood vessels, with stiffer probes having a higher incidence of damage. Depending on the location, stiffer probes may not compromise the BBB severely, as seen from the subset of silicon probes in this study that did not show an intense IgG level. However, a joint characterization of IgG with vasculature will help to confirm that the degree of IgG response is correlated to nearby vessels.

Unlike CD68, GFP, and IgG, silicon probes did not demonstrate more severe inflammation than the polymer probes in the context of GFAP and NeuN responses. We expected to see a decrease in GFAP intensity and an increase in neuronal density around the polymer probes, as studies suggest an inverse relationship of neuronal density to astrocytic scar, microglial activation, and BBB integrity in the chronic phase [46–48]. Along with the statistics, however, representative images of immunolabels that are approximately from the same region also suggested that probes with high CD68, GFP, and IgG did not always exhibit a significant astrogliosis or decrease in neuronal density. It should be noted that discrepancies among studies evaluating flexible probes [23, 24] indicate that differences in surgery, immunohistology, imaging, and analysis methods can impact the results more significantly than flexibility. Moreover, aforementioned studies were conducted in rats. Although not dramatically different from rats, mice are reported to show more prolonged astrocytic activation and higher neuronal density around implanted probes [49]. Taken together, previous studies are not directly comparable and discrepancies observed in this study need to be considered in their own context.

With GFAP intensity, there were significant differences between OSTE+Hard probes and polyimide probes at
4 WPI, and between silicon probes and OSTE+Soft probes at 8 WPI. However, these were inconsistent across the two time points and across the other immunolabels. With NeuN, no statistically significant differences in neuronal density were observed with any pair of probes at either time point. Although not statistically significant, neuronal densities at 8 WPI were generally higher than 4 WPI in the 0–264 µm region. This is in line with previous findings that neurons around an implanted device exhibit a multi-phasic response over time [23, 43, 50], the mechanism of which is yet to be understood as recording/stimulation performance typically does not recover at later time points. It is worth pointing out that with OSTE+Hard and OSTE+Soft probes we did not observe such ‘come back’ of neurons at 8 WPI. However, it is unlikely that this is a phenomenon pertaining to OSTE+ probes since there was no indication of OSTE+ being neurotoxic from the results of inflammatory markers. A cytotoxicity assay (MTT assay) in our previous study confirmed that OSTE+ samples were non-toxic after a sufficient incubation in MilliQ water [34]. The size of the OSTE+ samples used in the MTT assay were 1 mm thick, roughly 45 times thicker than OSTE+ probes. Based on simple diffusion, unreacted constituents in the 22 µm thick probe would be eliminated quicker than those in the 1 mm thick sample.

This suggest that OSTE+ probes are unlikely to chemically induce inflammation.

Surface chemistry, including cytotoxicity, hydrophobicity, and topology, is of critical concern regarding the biocompatibility of neural implants. These are known to affect the degree of protein adsorption and subsequent inflammatory response [54]. It is possible that differences in these characteristics may have influenced the outcome, although we do not believe that the impact was prominent. Silicon, polyimide, and OSTE+ probes used in this study all have smooth surfaces in sub-micron scale, previously revealed by scanning electron microscopy (SEM) [34, 55]. Not only the three polymer probes, but also silicon probes have been reported to maintain their structural integrity in vivo [55]. Only minor delamination of the silicon oxide traces was reported at 4 months and beyond [56], which may impact the electrical characteristics but not the biological response. Moreover, all four types of probe materials are hydrophobic and there was no drastic difference in their affinity to organic compounds. Along with the non-cytotoxicity of OSTE+, these suggest that the impact of surface chemistry could be marginal.

We preserved the probes within the tissue throughout the entire histology procedure. This mainly prevented tissue distortion during device extraction and allowed us to image

![Figure 6](image-url). Neuronal nuclei (NeuN) density near the implanted probes. (A) Representative fluorescent images of silicon, polyimide, OSTE+Hard and OSTE+Soft probes at 4 WPI and 8 WPI. (B) and (C) Normalized neuronal density as a function of distance with standard error. No statistically significant differences were found with Tukey’s pairwise comparison (p > 0.05). Scale bar is 100 µm.
the device-tissue interface more precisely. A drawback was that silicon devices mostly shattered while cryosectioning; however, it did not severely alter the interface as seen in the figures 3–6. Reflected fluorescence from the polymer probes was detected, with polyimide being the brightest followed by OSTE+Soft. This fluorescence further confirmed the precise location of the device. As a fluorescing device could interfere with our quantitative analysis, we took a conservative approach on the images to remove the device track prior to performing MINUTE analysis. Interestingly, we did not find any tissue slices with a hole that is far larger than the size of the device, which makes it hard to define the device contour for quantitative analysis.

A device’s flexibility depends on both the dimension and softness (i.e. Young’s modulus). In our study silicon probes were 15 µm thick and the three soft polymer probes were approximately 22 µm thick. As smaller probes are favorable in reducing the FBR as well as enhancing recording functionality [51], silicon devices were expected to cause a less severe FBR than the polymer probes if size was the sole variable. The difference of 7 µm in thickness means that silicon probes used in this study were softer (i.e. roughly 3 times in the planar direction (15:22 ≅ 1:3) and 1.5 times in the lateral direction (15:22 ≅ 1:1.5)) than the silicon probe with the same thickness as the polymer probes. Although silicon probes were thinner they induced the most severe FBR of the four types of probes, as indicated by CD68, GFP, and IgG intensities. The impact of stiffness of silicon probes likely overshadowed the benefit of small size. A simple dip-coating in PEG was chosen as the stiffening method for insertion of the polymer probes. Our initial test on a 0.85% agarose gel block revealed that OSTE+Hard and OSTE+Soft probes always buckled, and polyimide probes buckled by chance (8 out of 10 buckled). Dip-coating with 4 kDa PEG provided sufficient structural rigidity for penetration of the polymer probes. Inserting at a very high speed (100 mm s⁻¹) also helped the probes penetrate before the PEG dissolved in the cerebrospinal fluid. Higher insertion speeds have been reported to be beneficial in minimizing the shear force [52, 53] and this may have helped in minimizing the overall level of FBR in our study. No evidence of flexible probe deformation was found as probe shape and location along the depth (0–900 µm) were stable regardless of the probe type (data not shown). Note that silicon probes were also coated to prevent PEG-coating from confounding the study.

The fact that OSTE+ probes did not significantly reduce the FBR over polyimide probes suggest a threshold model for flexibility. This is further supported by OSTE+Soft probes not reducing the FBR over OSTE+Hard probes, negating the concern of comparing different materials. More evidence is required to conclude that the degree of FBR does not decrease with materials softer than OSTE+Soft. PDMS or even softer materials can be used to test this out, although challenges with fabricating and handling them remain to be solved. As depicted in figure 7, however, it is reasonable to speculate that the impact of flexibility in reducing the FBR would continue to diminish with increasing flexibility. A material’s Young’s modulus does not have to be as low as the interacting material to prevent generating significant strain stress as long as the material is sufficiently thin. Since the probes in this study were 15–22 µm thick, the results pertain to probes in this range of thickness, as well as other design parameters. Species and organ type can also affect the outcome as tissues have different softness and this can influence their tolerance level to the implant stiffness. It should also be noted that dummy devices used in this study were tethered to the silicon elastomer. If the device had a rigid wiring backbone, it can strengthen the degree of tetheredness to the skull and is another variable to be considered.

Resolving the biological tissue response to neural implants requires a multi-dimensional approach [6]. This includes flexibility, dimension, architecture, tethering to the skull, tissue integration, insertion profile, bioactive coatings, and drug release. One or more of these can be the limiting factor that dictates the eventual tissue response regardless of improvements in other aspects. Thus, we envision that the ‘threshold effect’, i.e. performance limits from softer devices, can be overcome once other factors are improved. Improving the

Figure 7. An illustrative graph showing hypothetical saturation of the impact of flexibility to the FBR.

Severe
Mild

Hard
Soft

Degree of FBR

Device Flexibility

Silicon
150 GPa

Polyimide
1.5 GPa

OSTE+Hard
300 MPa

OSTE+Soft
6 MPa

Brain
1 kPa

Hypothetical saturation curve in mice For devices 20 µm thick, 250 µm wide, 2 mm long

An illustrative graph showing hypothetical saturation of the impact of flexibility to the FBR.
biggest pitfall step by step will be key to advancing the life-time of neural implants.

5. Conclusion

We have investigated the biological impact of flexibility on the FBR by using four types of probes with different Young’s moduli: silicon, polyimide, OSTE+Hard and OSTE+Soft. A quantitative assessment of inflammatory biomarkers suggested that silicon probes induced the most severe FBR compared to the three soft polymer probes. No consistent statistical differences were found among the polymer probes, indicating that further increasing the probe flexibility beyond polyimide did not reduce the FBR. This suggest a possible threshold model for flexibility.

We believe OSTE+ probes are still a valuable addition to implantable electrode fabrication and designs as they performed no worse than polyimide probes. The tunable glass transition property and easy patternable nature of OSTE+ polymers can make them more useful over polydimethylsiloxane (PDMS), which is similarly soft to OSTE+Soft.

Acknowledgments

This project was sponsored by the Swedish Research Council (project # 80337401 and project # 60012701; 60012701 is a Linnaeus grant), by the Knut and Alice Wallenberg Foundation (project # KAW 2004.0119), by the University of Florida Preeminent Initiative Start-up Funds, and by the Defense Advanced Research Projects Agency (DARPA) Microsystems Technology Office (MTO), under the auspices of Dr Jack W Judy and Dr Douglas Weber (Pacific grant No. N66001-11-1-4013). We would like to thank Matilda Larsson at the Cen-...