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Amorphous Diamond In Vivo and In Vitro Evaluation

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Amorphous Diamond *In Vivo* and *In Vitro* Evaluation

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Abstract

This report describes the results of biocompatibility testing of amorphous diamond (aD), a Sandia-developed material that shows promise for bioMEMS applications. Material development and structure processing were performed at Sandia National Laboratories and biocompatibility testing, including *in vitro* studies (using cells grown in culture dishes) and *in vivo* studies (using aD coated Si die and aD microparticles implanted into mice), was performed at the Massachusetts Institute of Technology. *In vitro* studies were performed to evaluate the growth of 3T3 Fibroblast cells on bare aD surfaces, fibronectin-coated aD surfaces, and patterned aD surfaces, which consisted of dense arrays of aD islands ranging from 1 μm to 30 μm in diameter. Little cell growth was found on planar aD surfaces, while significantly greater cell growth was observed on the fibronectin-coated surface, though still less than the control surfaces of fibronectin-coated glass. The patterned surfaces also showed significant cell growth, with the cells showing preference for the edges of the etched aD. These studies suggest that the degree of cell growth on aD surfaces may be controllable, an important point for the development of unencapsulated *in vivo* sensors. *In vivo* studies were performed using aD coated Si die, approx. 5 mm × 5 mm × 0.5 mm, implanted into mice. The die and surrounding tissue were extracted at time periods ranging from 4 days to 6 mos. Very localized biological encapsulation of the die was found, and there was very little apparent inflammation or injury to adjacent tissues, suggesting a weak biological response. Some die showed evidence of aD film spallation with resulting particle release and capture by surrounding tissue. Whether this is related to a biological process leading to delamination or to poor adhesion in the initial fabrication of aD on the Si die is under current investigation. Regarding the *in vivo* response to aD disk-shaped particles of sizes ranging from 1 μm to 30 μm diameter, no evidence of inflammation or injury was found in the surrounding tissue following animal dissection and histological examination. The lack of biological response may be indicative of good material biocompatibility and/or the low total load of foreign material injected. In summary, the *in vivo* tests revealed no significant adverse biological reaction to the aD material, suggesting that future development of this material for bioMEMS applications may be promising.

Introduction

The contracted work was performed at MIT. Amorphous diamond (aD) is an interesting material as it has a very smooth surface, low stress, and high stiffness. The aim of the tasks was to evaluate the behavior of the aD *in vitro* - using cells grown in culture dishes - and *in vivo* - using aD coated silicon die implanted into mice - to evaluate the response to the material over 6 month terms - a long period for the evaluation of biocompatibility.

Methods

In Vitro Evaluation

To evaluate the in vitro (tissue culture) response of cells to the aD material, experiments were done with a common cell line, 3t3 Fibroblast cells. They were grown on aD coated silicon die and evaluated using LIVE/DEAD assay (from Molecular Probes). This assay combines two components to track both live and dead or damaged cells. Live cells have intracellular esterases that convert non-fluorescent, cell-permeable calcein acetoxymethyl (calcein AM) to the fluorescent compound calcein. Calcein is retained within cells - consequently, these cells fluoresce green on examination in a fluorescent microscope. Dead or damaged cells have permeable membranes; the second component, ethidium homodimer-1 (EthD-1) enters damaged cells and is fluorescent when bound to nucleic acids - this produces a bright red fluorescence in damaged or dead cells.

The first experiment was to coat die with an adhesion protein - fibronectin, and deposit cells on this surface and evaluate cell adhesion and cell viability using the LIVE/DEAD assay over the course of three days. Fibronectin adsorbs to many surfaces, and this protein assists in the initial adherence of the cells. After they have adhered, the 3T3 cells will start to produce their own extra cellular matrix, if the surface is suitable for long-term attachment. These samples were evaluated after one, two, and three days. The coating (adsorption) was done by pipeting a 20 μL drop of a solution of fibronectin (1 mg/mL - from Sigma) onto the surface and then covering this with a section of Parafilm to prevent evaporation. After one hour, the chips were washed 3x in sterile PBS and then 3T3 cells were seeded using standard techniques. The cells were grown in sterile conditions in a 37°C incubator with DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep (Penicillin - Streptamyacin) with 5% CO_2. All of the tissue culture supplies were purchased from Gibco.

Multiple samples were used for comparison, and different die were used for each time point. At the end of each time point, the die were fluorescently labeled following the instructions for the LIVE/DEAD assay kit (from Molecular Probes) and placed inverted on a glass slide with a drop of Vectashield mounting media (Vector) and imaged using a Zeiss Axiovert 200M inverted fluorescent microscope. Since the aD coated die are opaque, it was impossible to image them with a transmitted light mode such as phase contrast that is routinely used to image cells on transparent surfaces.

The second series of experiments was to evaluate cell adhesion and cell viability in the absence of supplemental extra cellular matrix (i.e. fibronectin). These samples were also evaluated after

one, two, and three days. Except for the coating with ECM, the methods are the same as those described above.

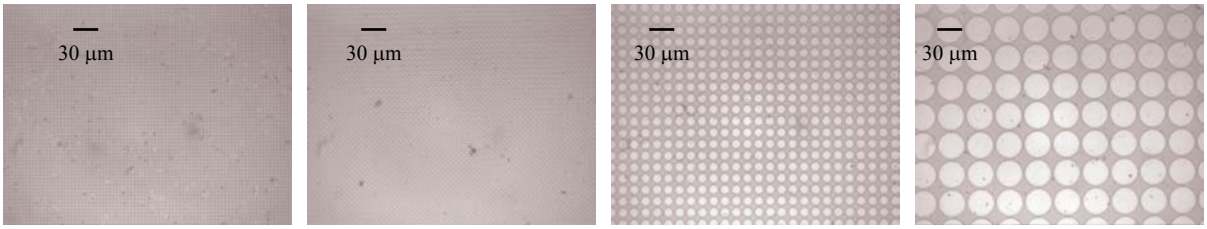
The final *in vitro* experiment was to compare adhesion on patterned surfaces. For these experiments, 3T3 cells were deposited onto a surface with aD regions of various geometries surrounded by a silicon (since it was exposed to water vapor and air, the surface must have a layer of SiO_2). One group of these die were cleaned with acetone, ethanol and then distilled water before being sterilized by five minute immersion in ethanol and 5x rinsing in PBS. The other group of samples were exposed to a 100W oxygen plasma cleaner, using air as the oxygen source, for 15 minutes. These die were handled and imaged in the same manner as described previously.

In Vivo Evaluation

All of the animal studies were done in a veterinarian supervised animal research facility and in accordance with The Guide for the Care and Use of Laboratory Animals (published by PHS-NIH) which complies with Animal Welfare Act and Public Health Service policy. The animal protocol and methods were approved by the MIT Committee on Animal Care (CAC).

To evaluate the biocompatibility of the aD coated silicon die, silicon die were prepared at Sandia coated on all surfaces with aD. The size of the die were 5mm x 5mm x 0.5mm. Male SV129 mice were used for these studies. The die had been cleaned by successive washes in acetone and ethanol, after which they were sterilized by washing for 5 minutes in ethanol, and then washed 5 times in sterile phosphate buffered saline (PBS) to remove the ethanol, just before implantation. These animals were anesthetized in a mixture of isoflurane and oxygen. After which, the backs of animals were shaved, then prepped in a sterile manner. A midline incision was made, and punches in the subcutaneous tissue were opened laterally by blunt dissection. aD coated die were placed within those pouches, such that they rested on the flank. At predetermined intervals (10 animals for each time point, time points at 4 days, 14 days, 1 month, 2 months, and 6 months), animals were euthanized by carbon dioxide and pentobarbital. The tissue capsule that formed around the implant was removed, placed in formalin, and processed for histology - before sectioning, the end of the capsule was removed by dissection, the die was carefully removed, and the capsule processed. Slides were made of sections of tissue cut at the midline of each die.

To evaluate the biocompatibility of the aD microparticles, male SV129 mice were also used. These animals were anesthetized in a mixture of isoflurane and oxygen. Animals were injected with a suspension of microparticles in 1% (w/v) sodium carboxymethylcellulose 0.1% (w/v) Tween 80 via a 22G needle. The injection was in the vicinity of the sciatic nerve at the level of the greater trochanter. The volume of particles injected in each animal was ~90 μg for 1 μm particles, ~200 μg for 3, 10, and 30 μm particles. At predetermined intervals (10 animals for each time point, 4 time points), animals were euthanized by carbon dioxide and pentobarbital. The tissue in the region of the injection was removed, placed in formalin, and processed for histology - multiple sections were mounted on slides to assist in finding the fine particles. Images of the die are shown in Figure 1.



(a) (b) (c) (d)

Figure 1. Particles of aD micromachined for biocompatibility studies - viewed in reflected light. (a) 1 micron, (b) 3 micron, (c) 10 micron, (d) 30 micron.

Results (the images below need to be viewed in color to interpret them)

***In vitro* analysis**

The results of the first round of experiments show that the surface of aD does not support cell adhesion in the absence of extra cellular matrix (ECM) and that with additional ECM to promote cell attachment, the surface does not encourage cell proliferation. Figure 2 shows results of cells that were deposited onto the surface with the additon of fibronectin and assayed after 1 day and 3 days. Each green spot is one cell. The image on the left shows a much greater number of cells on the surface of a die incubated for 24 hours than appear in the image taken (on a different sample) that was incubated for 3 days. Also, the image on the left shows an increased number of dead or damaged cells (red spots). For comparison, Figure 3 shows an image of a positive control sample, a glass coverslip also coated with fibronectin, is shown after incubating for 3 days - these cells have grown to confluence and show few dead or damaged cells.

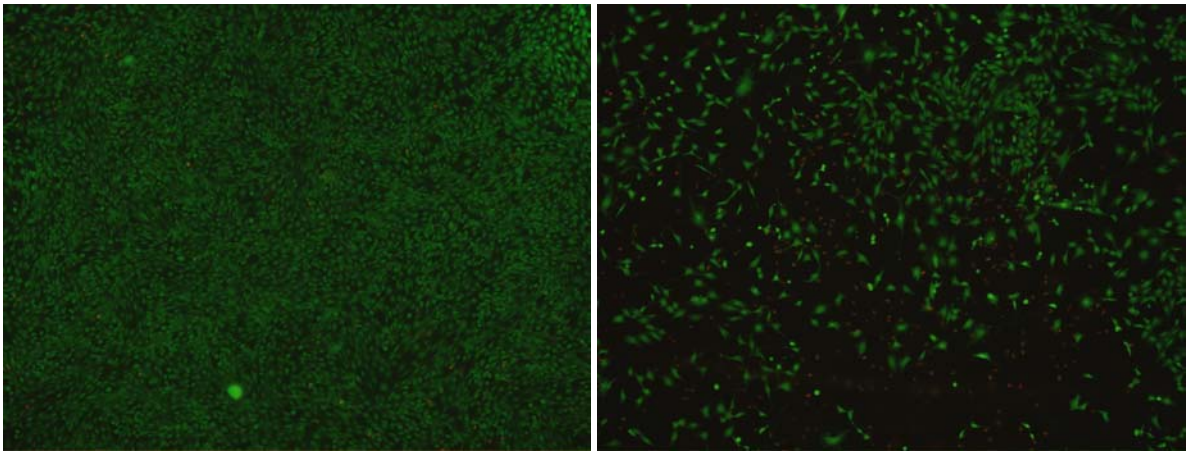

(a) (b)

Figure 2. 5x (a) Day 1, Fibronectin coated aD surface with 3T3 cells. (b) Day 3. Note the decreased number of cells after the third day, as well as the large number of red colored cells that indicate dead or damaged cells.

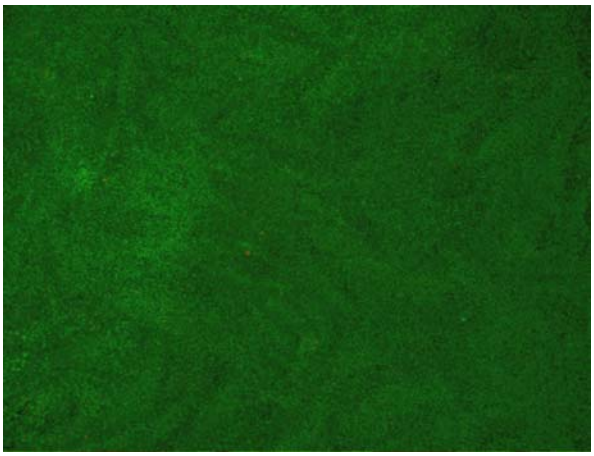
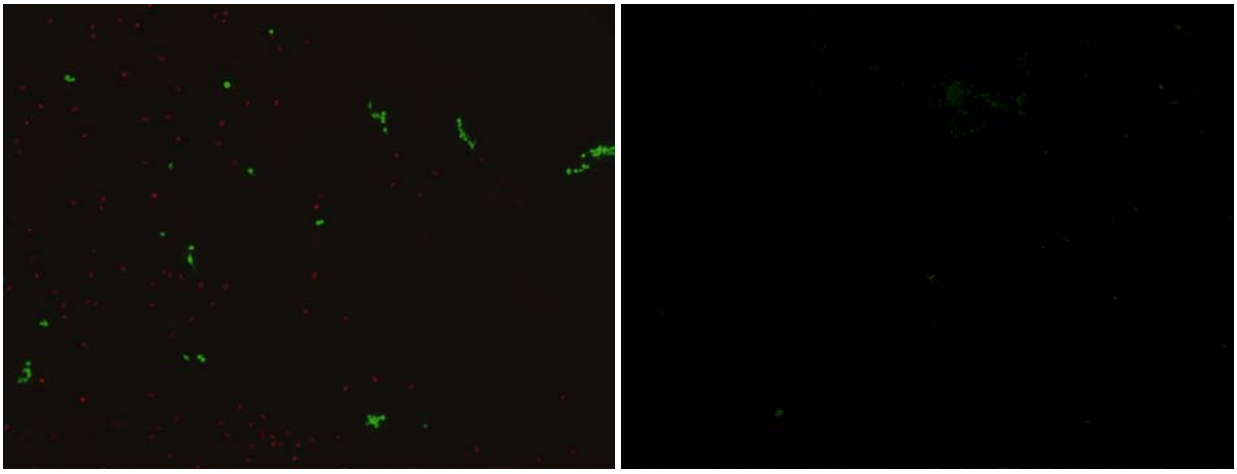


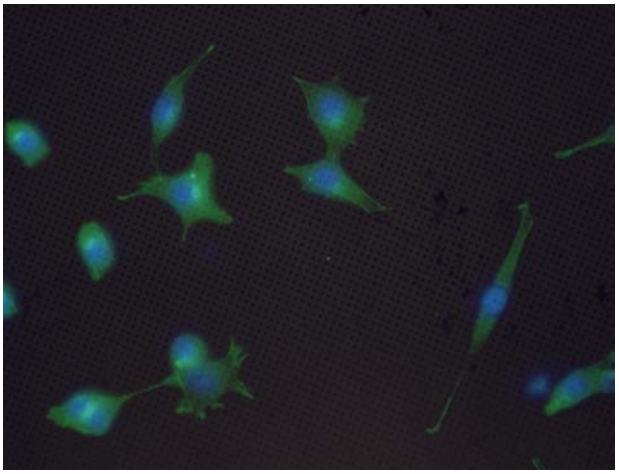
Figure 3. 5x Day 3 Fibronectin coated glass surface with 3T3 cells that have grown to confluence.



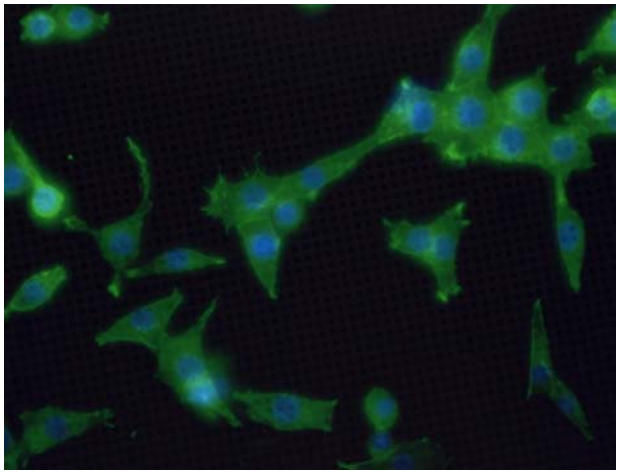
(a) (b)

Figure 4. 5x (a) Day 1, uncoated aD surface with 3T3 cells. (b) Day 3. Note the greatly decreased number of adherent 3T3 cells, and the large ratio of dead or damaged cells to healthy cells, and the absence of adherent 3T3 cells after 3 days.

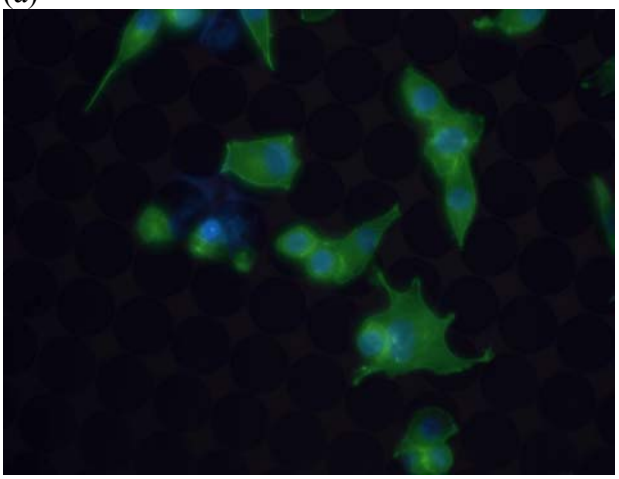
To evaluate the patterning of 3T3 cells using the properties of aD, circles of aD were patterned onto substrates with a background of silicon (the silicon surface would have formed at least a surface layer of SiO_2). Images from these experiments are shown below in Figure 5. In both sets of experiments, the cells were imaged for shape using a combination of phalloidin (stains actin in the cell cytoskeleton) and DAPI (stains the cell nucleus) dyes to highlight the cell morphology. There was not an obvious difference between the samples that were exposed to the mild oxygen plasma treatment and those that were not - in both cases the cells selectively adhered to the edges of the aD regions - suggesting possibly that the sp3 rich bulk material or the roughness of the material where it has been etched is preferable. It is interesting to note that more cells overall attached to these surfaces than to the homogenous aD surface used in the experiments shown in Figure 2 and 4.



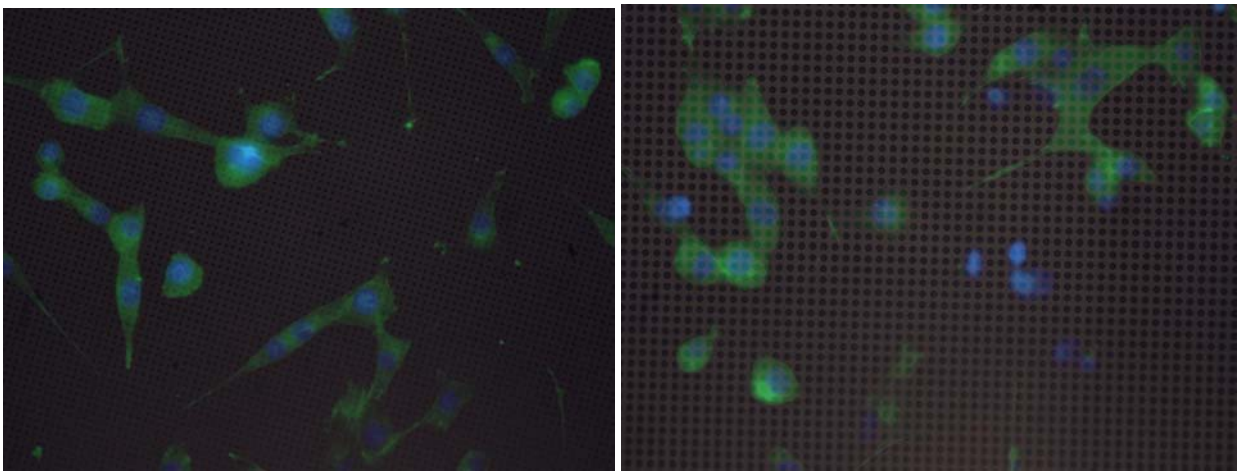
(a)



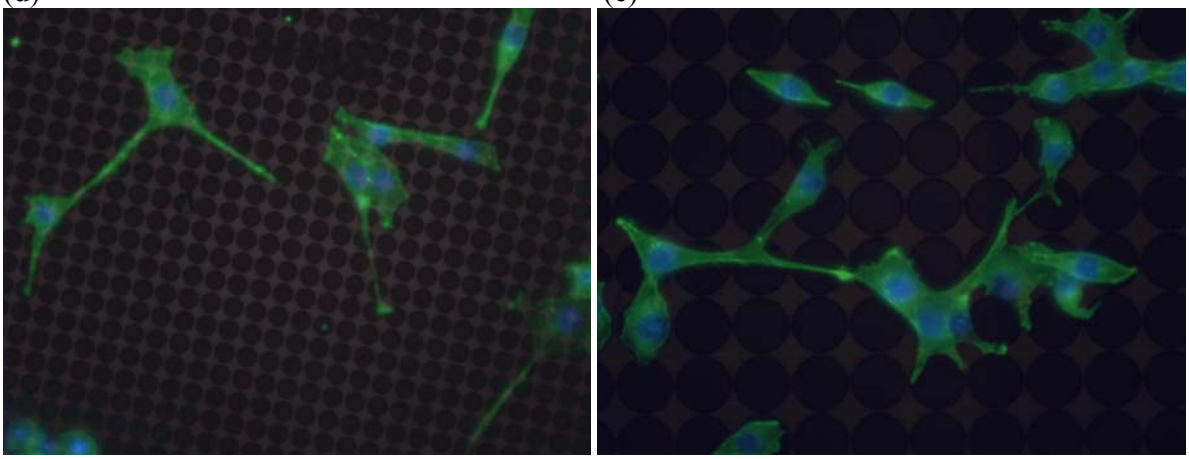
(b)



(c)



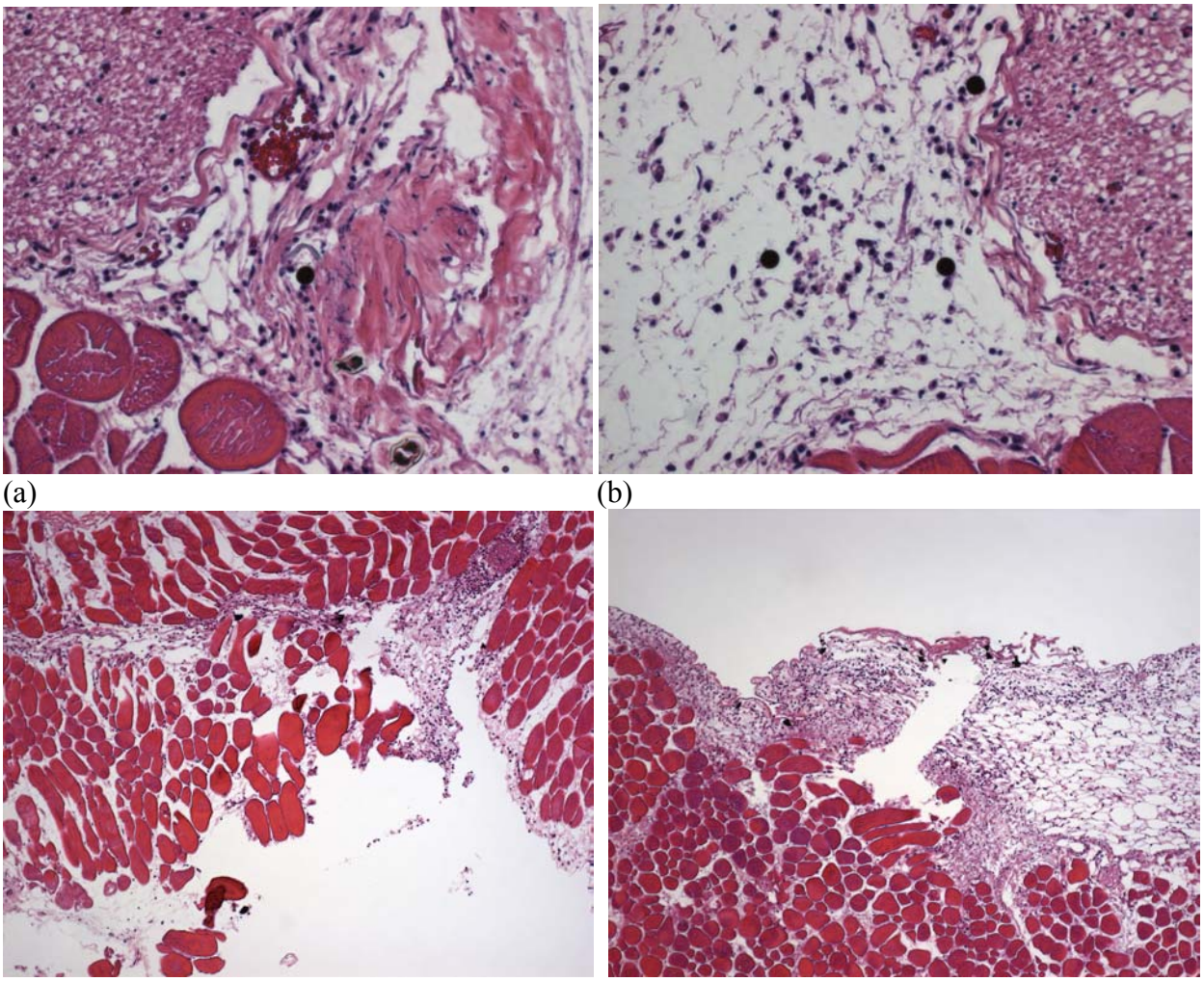
(d) (e)



(f) (g)

Figure 5. 40x images of patterned cells on (a-c) aD and silicon surface and (d-g) same surface plasma treated using a 100W plasma cleaner with air as an oxygen source and for 15 minutes. (a) 1 μm patterns (b) 3 μm patterns (c) 30 μm patterns (d) air plasma etched 1 μm patterns (e) air plasma etched 3 μm patterns (f) air plasma etched 10 μm patterns (g) air plasma etched 30 μm patterns

To evaluate the tissue response to aD particles, many sections were made through the harvested tissue in the vicinity of the sciatic nerve, where the aD particles were injected. There was no inflammatory or other adverse response to the aD particles which made it hard to find the exact site of the injection. Figure 6 shows some of the sections where particles were identified.

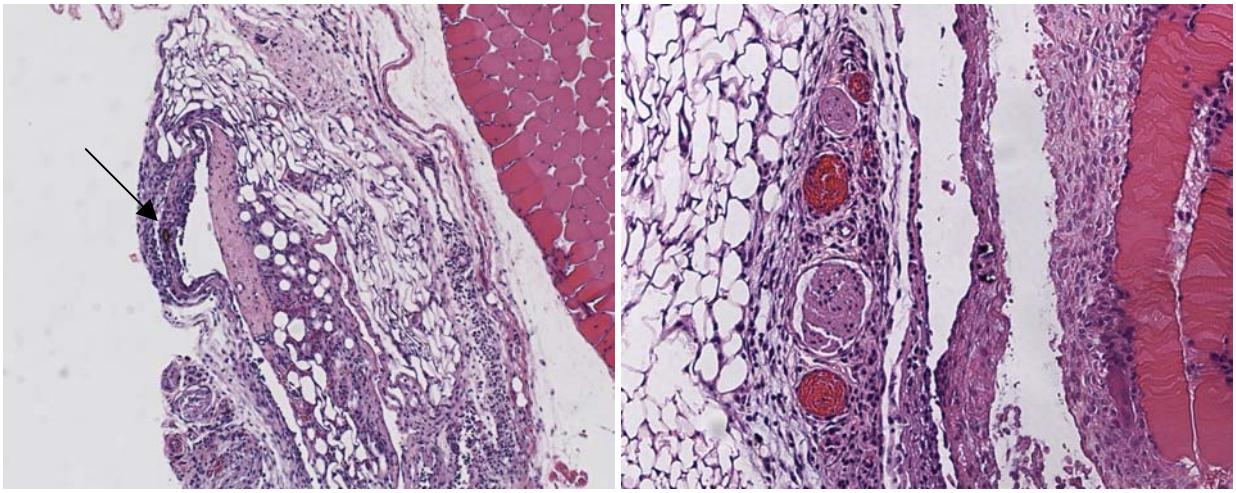


(a) (b)

(c) (d)

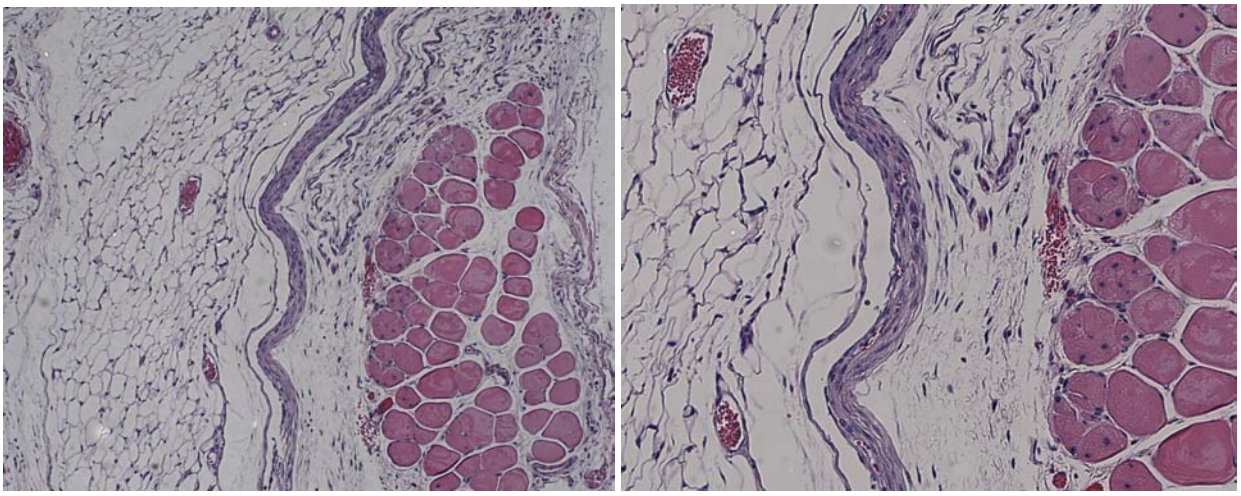
Figure 6. Sections of tissue showing 30 micron aD particles after sectioning. These samples were removed at the 1 month time point. No adverse effects are noted.(a) 40x (b) another region, 40x.(c) another region, 10x (d) another region, 10x

To evaluate the tissue response to aD coated silicon die, sections were made through the harvested tissue capsule that formed around the implanted die. Figures 7 through 12 show some of these sections at various time points. Figures 13 and 14 are reflected white light images of the explanted die after being removed from the tissue capsule - adherent cells are visible, as well as sections of the film that appear to have delaminated.



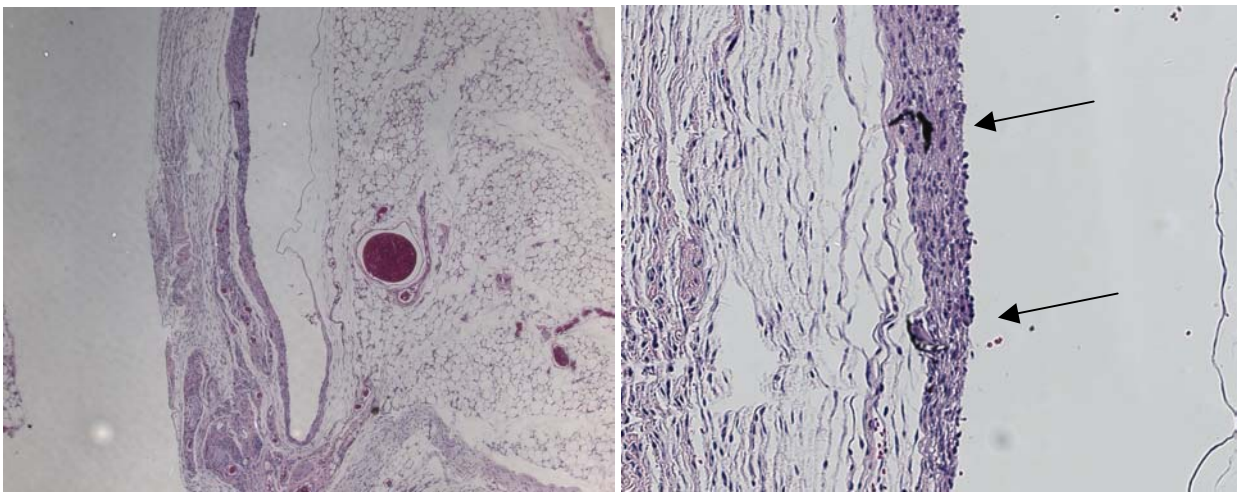
(a) (b)

Figure 7. Tissue capsules that formed around aD coated silicon die after 4 days. (a) 10x, (b) 10x. The arrow marks a fragment - this time point is too early to expect giant foreign body cells.



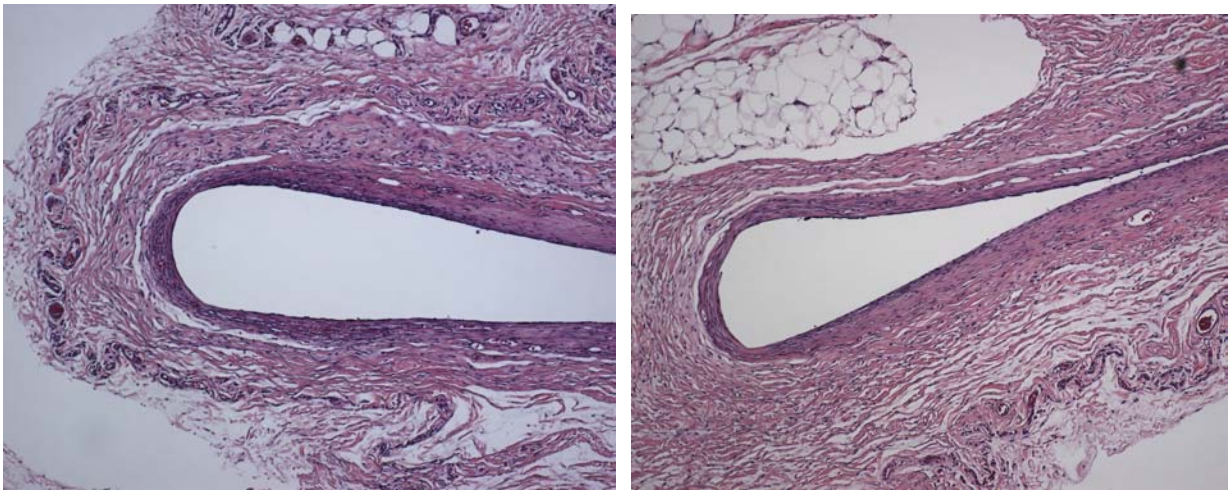
(a) (b)

Figure 8. Tissue capsules that formed around aD coated silicon die after 15 days. (a) 10x, (b) same region, 20x. The difference between the layer close to the muscle (right side) and the outer layer may be related to the mechanical disturbance of the moving muscle or it is possible that the outer layer adhered to the aD die when it was removed.



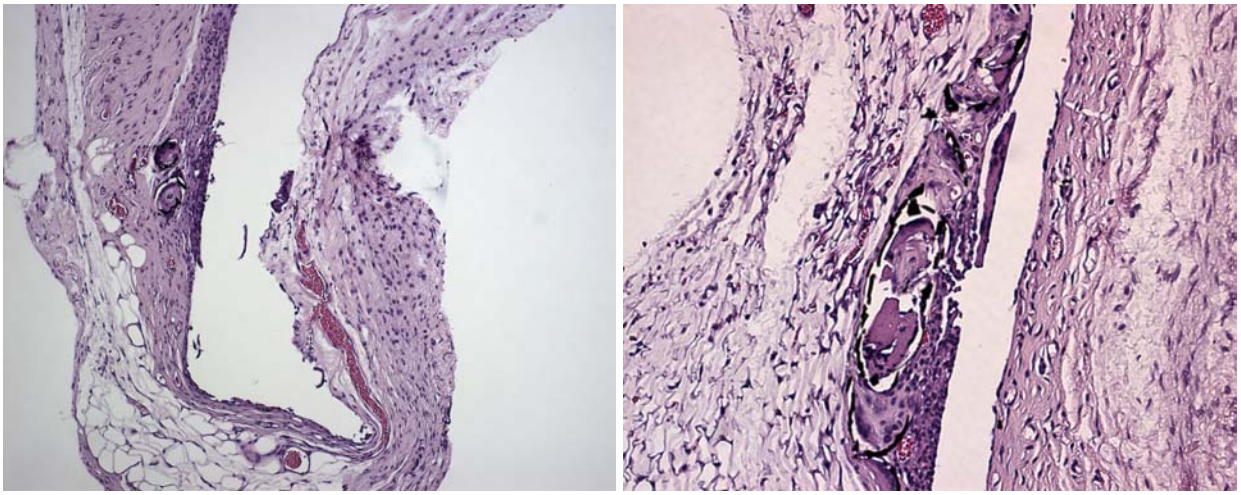
(a) (b)

Figure 9. Tissue capsules that formed around aD coated silicon die after 15 days. (a) 5x, (b) same region, 20x. The arrows marks fragments.



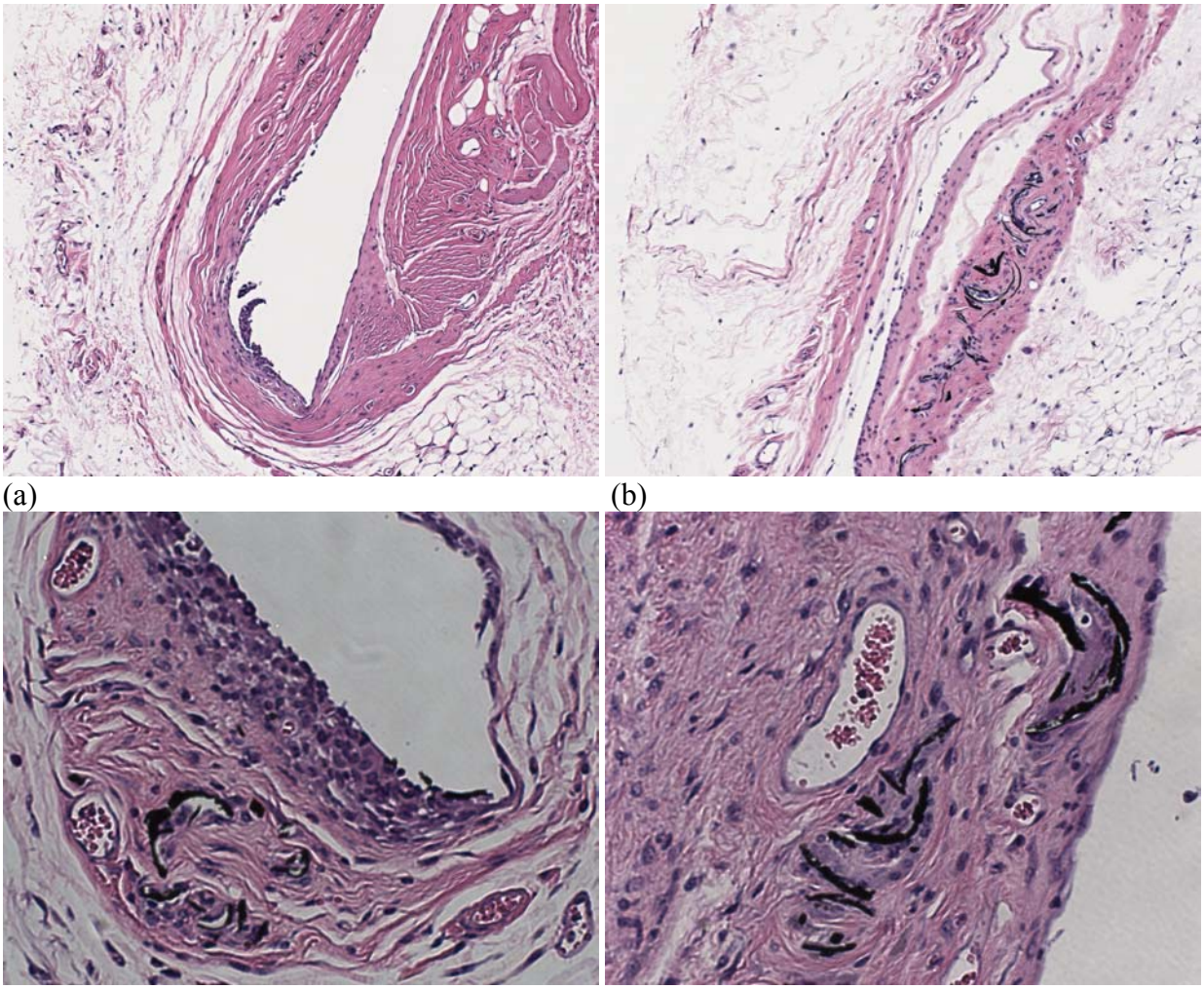
(a) (b)

Figure 10. Tissue capsules that formed around aD coated silicon die after 33 days. (a) 10x, (b) 10x.



(a) (b)

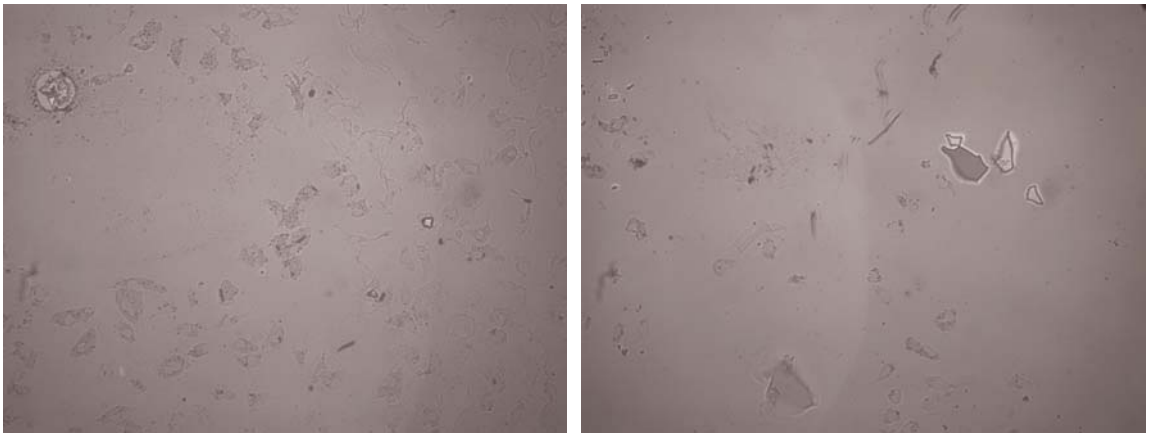
Figure 11. Tissue capsules that formed around aD coated silicon die after 2 months. (a) 10x, (b) 20x. There are many black colored fragments in the images. Note that these fragments appear buried in the tissue and do not appear to be the result of damage from the dissection procedure.



(a) (b)

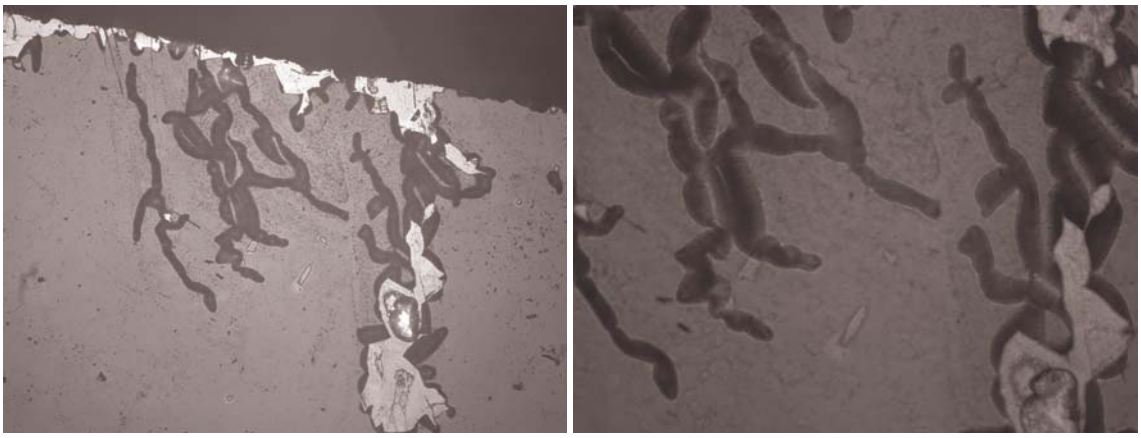
(c) (d)

Figure 12. Tissue capsules that formed around aD coated silicon die after 6 months. (a) 10x (b) 10x (c) 40x (d) 40x. There are many black colored fragments in the images. Note that these fragments appear buried in the tissue and do not appear to be the result of damage from the dissection procedure.



(a) (b)

Figure 13 (a) 6 month 20x - aD surface after explantation (b) different area, 40x



(a) (b)

Figure 14 6 month. Edge of aD surface after explantation - showing unusual film delamination (a) 20x (b) 40x (same area as (a))

Conclusions

In Vivo Biocompatibility of aD coated die

Dissection of animals into which wafers were implanted revealed a very localized encapsulation by tissue, with very little apparent inflammation or injury to adjacent tissues. The tissue surrounding the implant was not highly vascularized, nor inflamed. There was considerable variability within each time point in the thickness and opacity of the encapsulated tissue, such that we cannot point to a specific mechanism.

The tissue adherent to the wafers was removed and placed in formalin. The coated die were careful removed from the fixed tissue specimen in such as manner as to leave an intact capsule with a lumen (one end of the capsule had to be removed to access the die). On light microscopic examination of hematoxylin-eosin (H&E) stained sections of that tissue, the lumen was easily identifiable.

There were features that were common to all specimens, and of all time points examined - the lumen was generally surrounded by varying proportions of fibroblasts and inflammatory cells. The inflammatory response was chronic, featuring lymphocytes, macrophages, and giant multinucleate foreign body cells. There was generally a fibrotic response subjacent to these immune cells. On occasion, one side of the lumen had considerably greater cellularity than the other; it appeared that this might be related to closer proximity to a muscle, and perhaps therefore to greater frictional forces. It is possible that the samples in which the fibroblasts lined the lumen or where the cellular response was observed to be unusually mild compared to the other samples in the same time points reflect denudation of the lumenal surface by the process of die extrication. No conclusions were drawn from these occasional specimens. The chronic inflammatory response appeared increasingly robust with later time points, but was generally confined to a few cell layers surrounding the lumen; the surrounding tissues were unaffected.

There was one observation that was unique. In many samples, there were small isolated fragments of black opaque material, often with angular shapes that were in the vicinity of the lumen. These were interpreted as being aD debris. The fact that those debris were occasionally found within small clusters of inflammatory cells argues against the possibility that those fragments were the products of the wafer extrication process. Also, the fragments were seen in regions of sections corresponding to the edges of the die, as well as to regions of tissue corresponding to the centers of the die. On one section, an aD fragment was located in the lumen of a small blood vessel.

There did not appear to be any untoward biological sequelae in the vicinity of those fragments. None of the animals (10 per time point, time points at 4 days, 14 days, 1 month, 2 months, and 6 months) had an adverse event in the course of the study, and none appeared to show any indications of discomfort. However, the material being shed from the surface is a serious issue, as these fine particles, if they found their way into circulation, are large enough to obstruct a blood vessel which could possibly led to serious consequences. Because of our concern over this finding, additional samples were requested from Sandia, and some of the animal experiments were repeated - at no additional cost - to verify if the formation of the particles was the result of a

unique fabrication issue, or was typical for the material. These samples will be available for analysis in the beginning of March and an addendum to this report will be filed.

In Vivo Biocompatibility of aD particles

Dissection of animals into whom particles were injected revealed an entirely normal tissue appearance, with no evidence of injury or inflammation. Particles were only identified in a minority of histological sections. Where they were noted, there was no inflammatory response, even though some were of a size (30 μm) where the response of a giant foreign body giant cell might be expected. This lack of response may be related to the very small total load of material that was injected. No other adverse effects to the particles was noted.