

Chapter 1 Draft

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1 Introduction

according to the National Eye Institute (NEI), approximately 40000 cornea transplants are performed every year in the United States. Unfortunately, about 20 percent (8000 per year) of these transplants are rejected. Cite @ <http://www.nei.nih.gov/health/cornealdisease/> As a result, the recipient of the transplanted cornea experiences extreme discomfort and requires a second corneal transplant. Therefore, it is desirable to find ways to minimize the amount of rejection as well as increase the overall supply of tissue for cornea transplants.

Prof. Liz Orwin's lab at Harvey Mudd College is attempting to overcome the challenges of cornea transplants by growing replacement corneas. This is done by electrospinning collagen fibers, then implanting cells from a patient who needs new corneas. However, the electrospun collagen does not possess the structural properties of corneal collagen, so the Orwin Lab is focused on finding ways to control the phenotype and regulatory mechanisms of the cells. It is hoped that through this process, the cells will reenact the initial formation of the corneas in fetal development, taking up the electrospun collagen and laying it back down in a transparent form.

In order to better understand the behavior of the cells in response to the treatments they receive, Prof. Orwin's lab would like to be able to track the cells in the scaffold both by position and phenotype. Confocal microscopy cannot image deep enough into the scaffold to resolve all the cells, so the optical coherence microscope (OCM) was employed to track the cells, as its working distance (7 mm in air) is more than sufficient to image the scaffold.

Unfortunately, the collagen in the scaffold scatters too highly for the OCM to distinguish the cells, so Emily Hogan '07 developed a technique from the literature wherein 35 nm gold nanoparticles would be applied to increase scattering of the cells, called indirect immunolabeling, the basic precepts of which are still used in this project Cite . Indirect immunolabeling has four key components: a target protein (we are targeting $\alpha 5 \beta 1$ -integrin), a primary antibody that is *anti*- the target protein (MAB1999), a secondary antibody that is *anti*- the primary antibody (AP124F), and a label (both the gold nanoparticles and a fluorescent molecule on the AP124F). Indirect immunolabeling is implemented by first binding primary antibodies (Ab1) specific to surface proteins on corneal cells to those surface proteins. Then, secondary antibodies (anti-Ab1) are bound to nanoparticles via Van der Waals forces. The antibody-gold is then allowed to then bind to the Ab1-protein-cell *in vitro*.

Although this technique produced a statistically significant increase in scattering, the scattering increase was not as great as was desired. To address this, David Coats '08 did a number of studies to optimize the gold being used as the labeling agent. Coats performed

extensive calculations using Mie theory to maximize scattering. Mie theory details the scattering pattern of particles on the order of or larger than the wavelength of light they scatter. As even a 35 nm nanoparticle cannot be approximated as a point when using 850 nm light, Mie theory provides a far more accurate prediction of the intensity of backscattered intensity than Rayleigh scattering theory. These calculations showed that for any diameter, a particle made of solid gold (versus a particle with an inner core of silica) gave the highest absorption to scattering cross-section ratio, as shown in Figure 1. As can also be seen in that image, C_{sca}/C_{abs} also increases with diameter, up to a point. However, the settling rate of the gold particles also had to be taken into account, as well as the availability of manufactured gold nanoparticles. With these multiple factors in consideration, it was decided that 90 nm diameter nanoparticles from Nanopartz would be used. With these improved gold particles, David Coats again performed imaging tests on a monolayer of cells. However, the same lack of obvious signal generation prompted even further investigation.

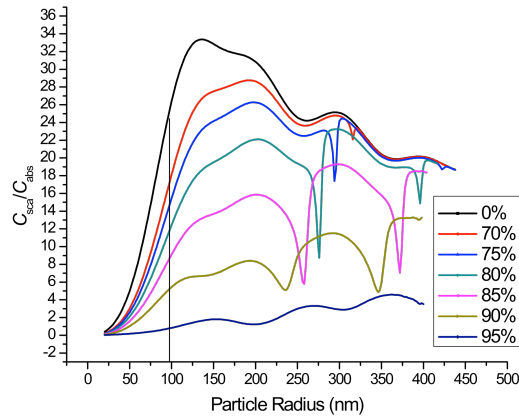


Figure 1: Plot of ratio of scattering cross section C_{sca} to absorption cross section C_{abs} as a function of total particle diameter, calculated via Mie theory. Line color, as indicated by legend, corresponds to percent of total diameter taken up by silica inner core. Index of refraction values used are $n_{Au} = 0.194 - 5.527i$, $n_{silica} = 1.44$, and $n_{water} = 1.33$ for the surrounding region.

One concern regarding the labeling system was that the antibodies were bound to the gold with only van der Waals forces. Rob Warren '10 discovered a procedure in the literature [cite] by which increased binding had been achieved for immunolabeling. The procedure consists of using a polyethylene glycol (PEG) polymer chain functionalized at one end with orthopyridyl disulfide (OPSS) and at the other with an N-Hydroxysuccinimide ester (NHS). The OPSS-PEG-NHS is incubated with the antibody for 24 hours, in which time the NHS ester reacts with a lysine in the antibody, leaving the NHS ester free in solution and the antibody attached to the OPSS-PEG. This OPAb, as we call it, is then incubated with the gold for 24 hours, so that the OPSS group splits at the disulfide bond and the PEG chain attaches to the gold via a thiol bond, which has a strength slightly less than that of a covalent bond and much larger than that of a van der Waals bond (appendix or footnote or reference on thiol bonds?). This should ensure that the antibody is firmly attached to the gold. The second part of the procedure is to prevent any other molecules from binding with

the gold. This is accomplished by binding another PEG chain to the gold, of which one end is functionalized with a thiol group (SH) that then forms a thiol bond with the gold. This should fill on any space left after the addition of the OPAb.

Unfortunately, these tests also failed to produce the desired specific increase in scattering, so in Summer 2010 Oliver Hoidn and Perry Ellis '11 undertook a reexamination of the PEGylation protocol. Several significant results emerged from their studies that indicated problems with the PEGylation protocol. First, the gold nanoparticle solution, though its pH is given as 7.1 (get actual number), was actually measured at pH 5.5 (get actual number) due to the lack of a buffer in the solution. However, if commercial PBS is added, the salt in that solution screens the negative capping agent on the gold nanoparticles, thus preventing the mutual repulsion between the nanoparticles and causing them to agglomerate and fall out of solution. To remedy this, a buffer solution made of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ was added to the gold to achieve a total salt concentration of 10 mM. This concentration was chosen as it provides a sufficient level of buffering without screening the capping agent too much. However, over the course of the several days required for the PEGylation process, it was found that the gold still suffered from a high rate of agglomeration. A footnote was then found in !cite that noted that PEG-SH cannot perform its protective function unless it has a molecular weight of at least 5 kDa. As the PEG-SH used up to that point had been of molecular weight 1.2 kDa, it had not protected the gold.

Thus, after Oliver and Perry concluded their work, it remained to readjust the PEGylation protocol for 5 kDa PEG-SH, and to use that updated protocol to once again try and get significantly specific contrast increase. To date, the PEGylation protocol has been optimized with the 5 kDa PEG-SH and produces results extremely consistent with the model of PEGylation we have developed. In addition, the 5 kDa PEG-SH appears to provide excellent long-term protection to the spheres. A study of binding to a monolayer of cells is to commence presently, and should that succeed, a 3D collagen matrix with embedded cells will then be tested.