

Counteracts: Non-invasive Prevention and Treatment of Cataracts

Author contributions:

Conceptualization, F.T., H.H., M.S., C.S.L., A.W., A.E.W., M.Y., D.J., L.Y., A.L., A.Y.L., J.C., H.J., A.L., J.C., K.L., J.Y., C.Y., Y.W., J.L., A.C., Z.V., and S.C. Methodology, A.W., F.T., H.H., and A.E.W.; Software, A.W. Investigation, F.T., H.H., M.S., C.S.L., A.W., A.E.W., M.Y., D.J., L.Y., A.L., A.Y.L., J.C., H.J., A.L., J.C., K.L., J.Y., C.Y., Y.W., J.L., A.C., Z.V., and S.C. Resources, J.C. and T.C.; Writing – Original Draft, F.T., H.H., M.S., C.L., A.W., M.Y., D.J., and A.E.W.; Writing – Review & Editing, F.T., H.H., M.S., C.L., A.W., M.Y., D.J., and A.E.W.; Visualization, J.Y. and A.W.; Supervision, J.C. and T.C.; Project Administration, J.C.; Funding Acquisition, J.C.

Abstract:

Cataract, the clouding of the lens, causes half of the world's blindness. Most cataracts are age-related and arise when crystallin proteins in the lens become oxidized and aggregate over time. Surgery is currently the only recognized treatment to effectively cure cataracts, but this method is expensive and invasive. Therefore, we formulated a natural and non-invasive alternative. In the eye, a natural antioxidant glutathione—produced by the enzyme glutathione reductase—exists to combat oxidation, but GSH levels decrease with age. Studies have also suggested that another enzyme—25-hydroxylase—can convert cholesterol in the eye into 25-hydroxycholesterol, which reverses crystallin aggregation. We expressed glutathione reductase and 25-hydroxylase for the prevention and treatment of cataracts, respectively. To purify the proteins, we included a 6x-polyhistidine tag and tested that proteins can be successfully isolated from the bacteria. To deliver these proteins through the cornea and into the lens, we engineered biodegradable chitosan nanoparticles. Our nanoparticles encapsulation and protein release data suggest a natural and non-invasive alternative to surgery.

Introduction:

Cataracts are the leading cause of blindness today, affecting 20 million people worldwide (World Health Organization). Half of Americans above 80 years old are affected by cataracts (National Eye Institute). The National Eye Institute projects that in 30 years, the number of cataract patients will increase to 50 million (National Eye Institute). However, the current standard treatment for cataracts is invasive and requires trained surgeons with professional equipment. These requirements add to the cost of surgery, which averages about \$3,500 per eye in the US without insurance (Segre, 2016).

Comments

jejohnston: You'll want to change your reference style to Vancouver.
alvinwang: Thank you for your comment! We've changed our reference style to Vancouver.

Cataracts can be caused by many factors, including radiation and diabetes, but the underlying cause is oxidative damage. Oxidative damage happens when reactive oxygen species, or unstable chemicals containing oxygen, react with DNA, lipids, or proteins, disrupting cellular functions (Truscott, 2005). In the lens, crystallin proteins can be oxidized by hydrogen peroxide (H_2O_2), a reactive molecule produced during aerobic respiration (Giorgio et al., 2007). H_2O_2 reacts with protein residues and changes the shape of the protein. The damaged proteins thus aggregate and form clumps in the lens (Truscott, 2005). In the eye, a natural antioxidant called glutathione (GSH) exists, which converts H_2O_2 into water (Giblin, 2000). With age, however, GSH level decreases, meaning that less H_2O_2 is being converted to water, and oxidative damage caused by H_2O_2 increases. Overtime, buildup of oxidized proteins will lead to the development of cataracts. When GSH becomes oxidized, a disulfide bond forms between two GSH molecules, turning it into oxidized glutathione (GSSG).

Our aim is to develop noninvasive, easy-to-use, and affordable eye drops to prevent and treat cataracts. For prevention, we engineered a construct that codes for GSR, the enzyme that recycles GSSG back into GSH. For treatment, we engineered a construct expressing cholesterol 25-hydroxylase (CH25H). CH25H converts cholesterol into 25-hydroxycholesterol (25HC), a molecule that restores solubility of protein clumps and lens transparency (Makley et al., 2015). We then tested the effects of GSH and 25HC in a fish lens cataracts model. To non-invasively deliver proteins that produce these molecules in the lens, we synthesized chitosan nanoparticles as protein carriers to the lens (Gan et al., 2007). We then tested protein encapsulation and release of chitosan nanoparticles.

Materials and Methods:

Setting up a cataract model

We purchased dead *Priacanthus macracanthus* and extracted soluble proteins from the lens. We removed the lens cortex and obtained the nucleus because the nucleus contains older cells and is more prone to cataract formation (Cvekl & Ashery-Padan, 2014). In order to isolate the lens protein, we shook the lens nucleus in Tris buffer overnight and centrifuged the samples (Mello et al., 2012) (Figure 1). We then extracted the supernatant, which contains soluble proteins from the lens nucleus and added hydrogen peroxide (H_2O_2) until the desired concentration was reached.

Comments

ddelphine: What is the "desired concentration" or the range of concentration that you tested?
jpgoupil: Identifying where you got the *Priacanthus macracanthus* would be helpful; it's generally good practice to say where you got things to enhance reproducibility
alvinwang: Thank you for your comments! We've updated the section to include the concentrations (0, 1, and 10 mM) and where we purchased the fish.

Measuring absorbance

To quantify the severity of cataracts in our model, we used a spectrophotometer to measure absorbance values of the lens solution. As lens proteins become oxidized, absorbance should increase because as proteins aggregate, they fall out of solution, which will scatter incoming light. To find a wavelength of light for data collection, we compared the absorbance of lens solution alone (negative control), H_2O_2 -treated lens solution, and heat-denatured lens solution (positive control). We observed an absorbance peak at 397.5 nm for both H_2O_2 -treated and heat-denatured solutions, and chose to collect all future absorbance values at that wavelength. Using SDS-page, we confirmed that the H_2O_2 -treated lens solution exhibited protein aggregation (Figure 2).

Comments

archisrb: What is the concentration of the H_2O_2 solution used to treat the lens in this figure? I don't think it is specified in the manuscript
alvinwang: Thank you for your comment! We've updated it to include the concentration (10 mM).

Prevention of cataracts with glutathione:

We purchased GSH from Sigma Aldrich. We treated 3 samples lens solution (3 mL each) with 8 mg GSH then added H₂O₂ until to reach 10 mM H₂O₂. For negative control, we only added H₂O₂ and not GSH. We measured the absorbance of the solutions at 24-hour increments (Figure 3).

Comments

ddelphine: Little grammar mistake "until reaching" instead of "until to reach"
alvinwang: Thank you for your comment! We've changed the wording.

Treatment of cataracts with 25HC:

We purchased 25HC from Sigma Aldrich and pet eye drops from OcluVet. We dissolved the 25HC in ethanol by vortexing. We incubated lens solutions with H₂O₂ for 24 hours then added the 25HC solution to reach 20 μM 25HC and 50 μM 25HC. We also added 3 drops of pet eye drops for comparison. We measured the absorbance of the samples in 24hr increments (Figure 4).

Comments

ddelphine: What was the concentration of H₂O₂ used here? I suppose it's the same than the one used for controlling the effect of GSH on prevention of cataract but it could be useful to write it again there. And what was the purpose of testing the OcluVet eye drops? What are their effects on cataract?
ddelphine: Also, I think you wanted to reach 20μM 25HC OR 50 μM 25HC (not the 2 at the same time but in different solution I think).
alvinwang: Thank you for your comments! We've update it to include the concentration of H₂O₂ (10 mM). We tested the eye drops to compare 25HC with a commercially available product. We've also changed the wording from "and" to "or".

Construct design

We designed a construct including a strong promoter, strong ribosome binding site, GSR (mutated to remove internal restriction sites), 10x histidine-tag, and a double terminator (Figure 5). We acquired a strong promoter + strong ribosome binding site part (BBa_K880005) from the iGEM distribution kit to maximize protein production. We ordered the cDNA of GSR from Origene and used the 10x histidine-tag part from the distribution kit (BBa_K844000). A double terminator (BBa_B0015) was added at the end to stop transcription. The purchased GSR cDNA had two internal PstI and three EcoRI cutting sites. After making silent mutations to the sequence, we sent the cDNA to Mission Biotech for mutagenesis to remove these cutting sites. Once we had the correct sequence of GSR (with 5 point mutations), we designed primers to add the BioBrick prefix and suffix in order to clone GSR into a BioBrick backbone. The primer designs were sent to Tri-I Biotech for oligo synthesis, and polymerase chain reaction (PCR) was set up. We first cloned GSR behind BBa_K880005 (strong promoter + strong ribosome binding site; Figure 6), then front-inserted this before a new plasmid (Figure 7) containing 10x his-tag (BBa_K844000) and double terminator (BBa_B0015). Sequencing results from Tri-I Biotech show that our final construct was correct.

Comments

jejohnston: "...cloned GSR behind BBa_K880005..." You could rephrase this by saying GSR was placed under the control of a strong promoter and ribosome binding site.
alvinwang: Thank you for your comment! We've phrased the wording to reflect the steps taken to build the GSR construct.

We also designed a construct containing similar components as the prevention construct, except the first part of the open reading frame is replaced by CH25H (Figure 8). CH25H was also mutated (by Mission Biotech) to remove internal cutting sites and put into a Biobrick backbone to make a new basic part. The sequence of the final construct was sent to Integrated DNA Technologies for synthesis and cloned into a Biobrick backbone.

Chitosan nanoparticle synthesis

We dissolved 3 mg/mL low molecular weight chitosan in 1% (by volume) glacial acetic acid. We adjusted the pH of the chitosan solution to 5.5 by adding 1M sodium hydroxide, then dissolved 4 mg of desired protein for each millimeter of chitosan solution. We added proteins including bovine serum albumin (BSA), green fluorescent protein, red fluorescent protein, and green pigment protein (Figure 9). We dissolved 1 mg/mL of sodium triphosphate in water. We stirred the chitosan solution at 600 rpm while adding an equal volume of the sodium triphosphate solution drop wise. To isolate the nanoparticles, we centrifuged the nanoparticle suspension at 17000 xg for 40 minutes at 4 degrees Celsius. We imaged the pellet using atomic force microscopy to confirm nanoparticle formation (Figure 10).

Protein encapsulation measurement

We synthesized chitosan nanoparticles with and without bovine serum albumin (BSA), and collected the supernatants after centrifugation. We added known amounts of BSA to the blank supernatant to create standard solutions. Using a Bradford assay (Bio-Rad), we then measured the protein concentration in the supernatant of BSA-containing nanoparticles to measure the amount of proteins that failed to be encapsulated (Figure 11). To calculate encapsulation efficiency, we subtracted the measured protein concentration from 2, then divided by 2, since we added 2 mg/mL of BSA during nanoparticle synthesis.

Comments

jejohnston: The description of encapsulation efficiency was not clear. Also, did you use a kit from Bio-Rad for the Bradford assay, if yes, you should make that more explicit.
ddelphine: I didn't understand your last phrase about the calculation of encapsulation efficiency.
alvinwang: Thank you for your comments! We did not use a kit, but we did use Coomassie Blue purchased from Bio-Rad to perform the Bradford assay. We've rephrased the explanation of encapsulation efficiency and added an example to hopefully clarify our method.

Protein release measurement

We synthesized and isolated chitosan nanoparticles containing BSA. We resuspended the nanoparticles in phosphate buffered saline (Wilson et al., 2014). We placed the samples at two different temperatures: 4°C and 37°C. As the nanoparticles degraded, they released proteins into the saline solution. Using a Bradford assay, we measured protein

at two different temperatures, 4 °C and 37 °C. As the nanoparticles degraded, they released proteins into the saline solution. Using a Bradford assay, we measured protein concentration of the saline solution surrounding the nanoparticles over a 72-hour period (Figure 12)

Comments

jejohnston: You can remove the first sentence as it is redundant.

alvinwang: Thank you for your comment! We removed it.

Long-term nanoparticle model

We cannot experimentally test the behavior of our nanoparticle treatment over a long period, so we built a mathematical model to analyze the amount of GSR maintained in the lens over time, as eye drops are regularly applied. First, we create a model of the release of a single dose of nanoparticles (Figure 13), using the Noyes-Whitney equation (Fraunfelder et al., 2008). We sum up the contributions of each dose of nanoparticles which correspond to each application of the eye drop, yielding the final model of GSR concentration over time. We can alter variables, such as concentration, eye drop frequency, and nanoparticle radius, and analyze its impact on GSR concentration in the lens.

Chitosan nanoparticle-embedded contact lens

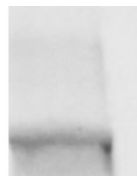
We found a method to make chitosan nanoparticle-embedded hydrogel contact lenses (Behl, 2016). Following their protocol, we created a polymer solution containing all the necessary components. We dispersed GFP-nanoparticles in the polymer solution to test if protein containing nanoparticles can be embedded in the contact lenses. We then transferred this solution into a 3D-printed mold (Figure 14, left). After exposure to UV for 40 minutes, we successfully made hydrogel contact lenses (Figure 14, right).

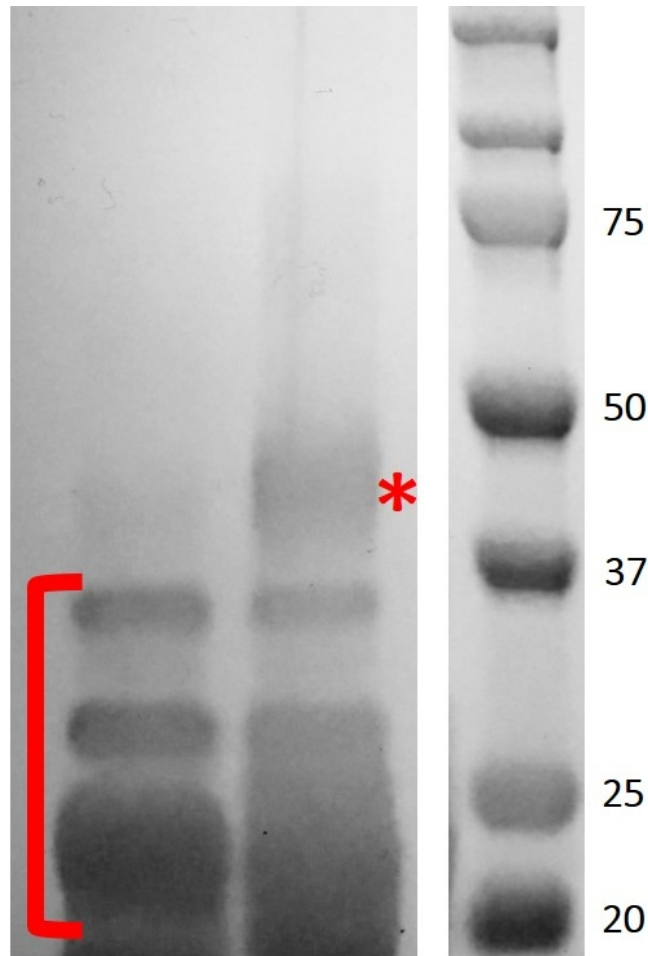
Data:



Figure 1. Our cataract model was set up using fish lens. Left: Priacanthus macracanthus purchased from the market. Right: the two spheres on the left are entire lenses and the two smaller spheres on the right are lens nuclei.

Fish Lens Proteins Fish Lens Proteins + H₂O₂





Comments

jejohnston: What protein ladder did you use?

alvinwang: Thank you for your comment! We've added information about the protein ladder used.

Figure 2. H_2O_2 causes protein aggregation. We ran a protein gel with our fish lens solutions, with or without H_2O_2 . Compared to the untreated solution, treatment with H_2O_2 resulted in higher bands (red asterisk). The right lane is the molecular ladder (showing protein sizes in kDa).

Comments

ddelphine: Several things missing there. You should clearly write in the legend that it is a SDS-PAGE (if I am not mistaken), so you are in denaturing conditions and the percentage of acrylamide should be make clear. You should also add the complete gel in supplementary data or something since cut gels like this one are not really trustworthy.

alvinwang: Thank you for your comment! We've added that it is a SDS-PAGE using 10% acrylamide. We will include the complete image in the supplementary information.



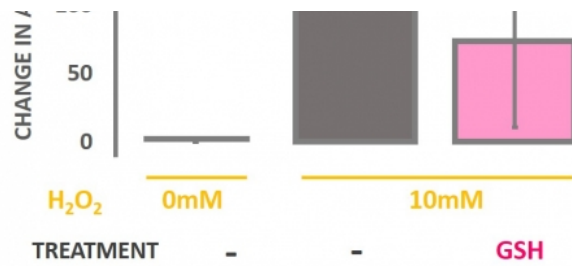


Figure 3. Addition of GSH results in a smaller increase in lens solution absorbance. Lens solutions were treated with H₂O₂ only, or with H₂O₂ and GSH together. Graph shows percent change in absorbance after 48 hours. Data from Table 1.

Comments

archisrb: Are the error bars here 95% confidence intervals or standard deviations? I think you should also specify n, the number of cataracts experimented on in the control and H₂O₂ treated groups.
alvinwang: Thank you for your comment! We've included that the error bars indicate standard error and the data reflects the average of two trials, which are included in the supplement.

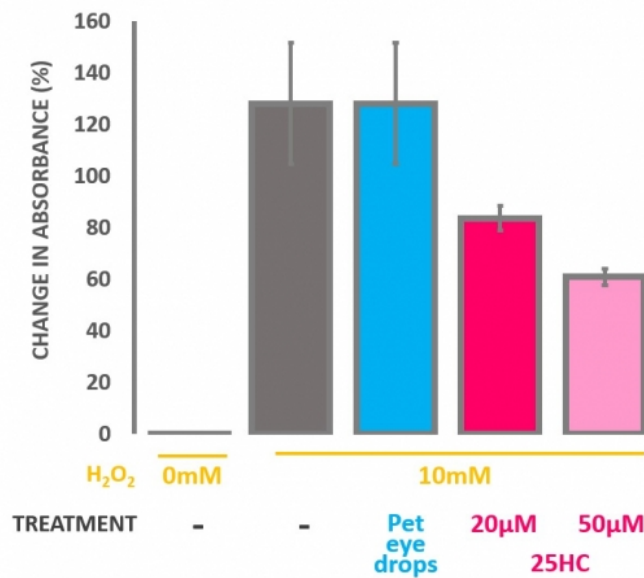


Figure 4. 25HC is more effective at treating cataracts compared to pet eye drops. Increasing the concentration of 25HC increases effectiveness. Two trials were conducted and the error bars showed a significant difference between untreated and 25HC-treated samples. Data from Table 2.

Comments

archisrb: Similar comment as I had in figure 3: I think you should specify n, the number of cataracts in the control and H₂O₂ treated groups.
ddelphine: I think you should rephrase the end of the legend. It is the fact that the error bars are really small for the treated samples compared to the untreated (or treated with pet eye drops) ones that emphasized the difference, not the error bar in itself.
alvinwang: Thank you for you comments! We caught an error in the graph, and we've updated the graph and its legend. The data (from two trials) can be found in the supplement.

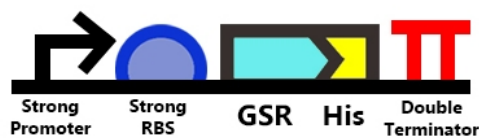
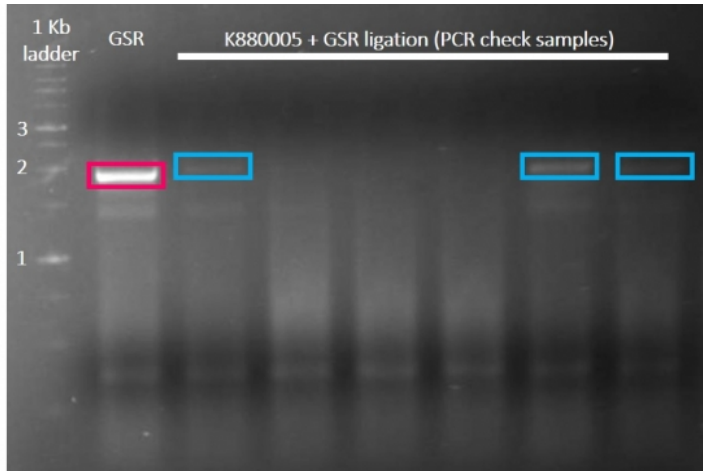


Figure 5. Our construct includes a strong promoter, strong ribosome binding site, GSR, his-tag, and double terminator.

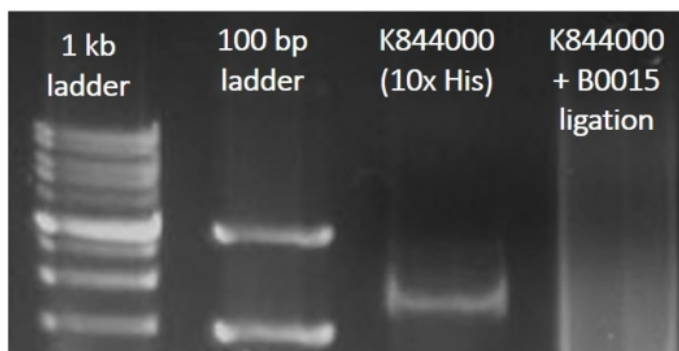


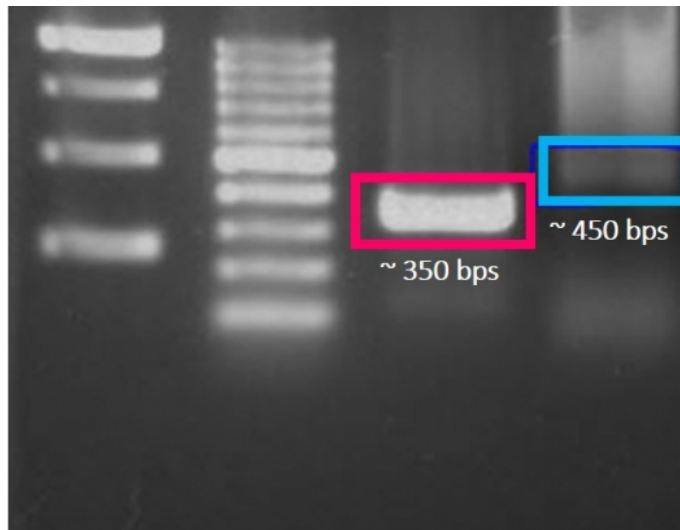
Comments

jejohnston: Your ladder labels need to be more clear. I assume you mean 1, 2, and 3Kb but you need to make it explicit.

alvinwang: Thank you for your comment! We've added the units in the legend.

Figure 6. PCR check for BBa_K880005 + GSR. After inserting GSR (pink box, 1.7kb) behind a strong promoter and strong RBS (BBa_K880005), the expected ligation size is around 2kb (blue box).



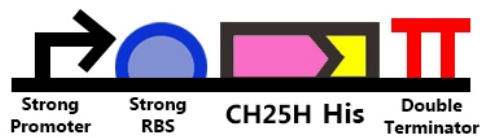


Comments

jejohnston: Same comment that I made about figure 6. If you are going to say how large a specific band is in your gel, you need to label your ladder so that people can see how large the bands are.

alvinwang: Thank you for your comment! We've labelled the ladder with the corresponding sizes.

Figure 7. PCR check for his-tag (BBa_K844000) + double terminator (BBa_B0015). The double terminator (~130 bps; or ~400 bps after PCR check, pink box) was inserted behind 10x his-tag (~30 bps). After PCR, the expected ligation size is ~450 bps (blue box).



Comments

ddelphine: I think you can merge this figure and the figure 5 to obtain one figure that show your two constructs (like figure 5A the one showing the construct for GSR and figure 5B showing the CH25H construct).

alvinwang: Thank you for your comment! We've merged the two figures.

Figure 8. Our construct includes a strong promoter, strong ribosome binding site, CH25H, his-tag, and double terminator.

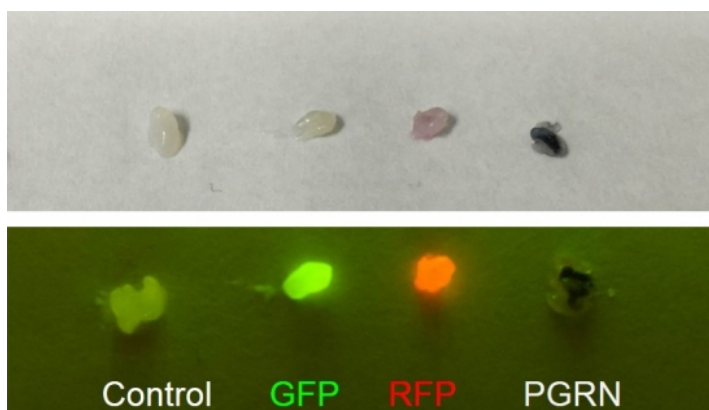


Figure 9. Proteins were successfully encapsulated into nanoparticles. Figure shows nanoparticle pellets containing no protein, GFP, RFP, and pGRN (left to right) under white light (top) and blue light (bottom). Fluorescence of GFP and RFP-containing pellets shows that proteins are still functional.

Comments

ddelphine: You forgot to introduce the abbreviations (GFP, RFP and pGRN) before in your Material and Methods. GFP and RFP are well-known proteins but pGRN is less common, so you remind there the meaning.

alvinwang: Thank you for your comment! We've introduced the abbreviations in the method section.

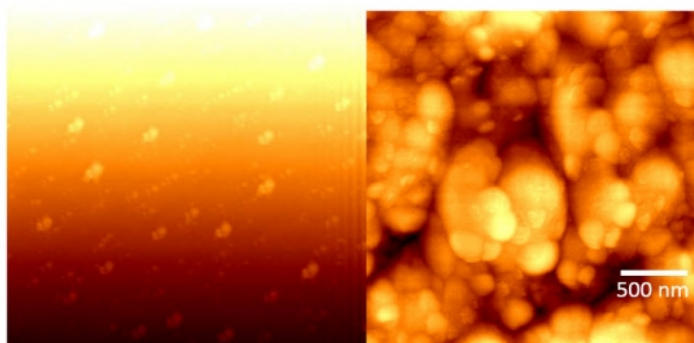


Figure 10. We imaged chitosan nanoparticles using atomic force microscopy. On the left is the empty silica plate. On the right is an image of the chitosan nanoparticles, which were placed on the silica plate.

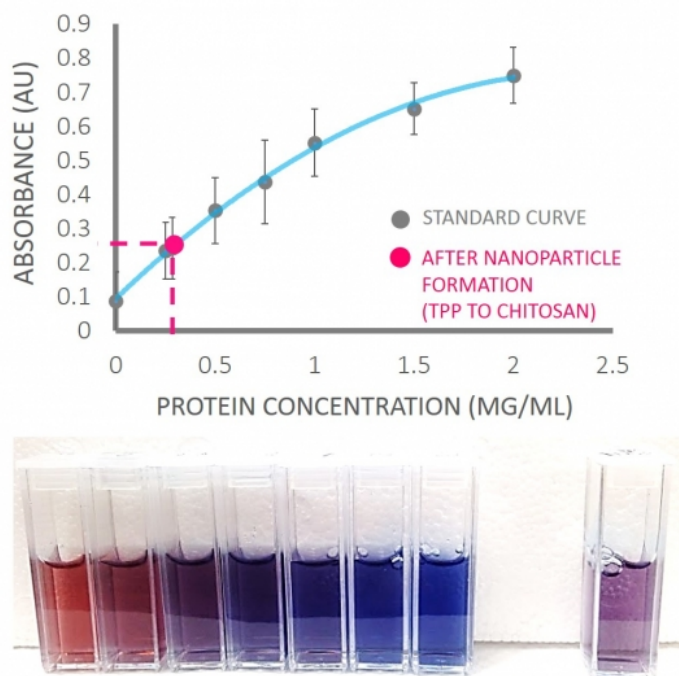
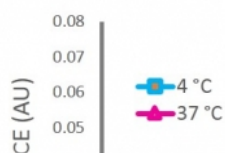


Figure 11. The encapsulation efficiency is 72%. Using a Bradford assay, we created a standard curve of known BSA protein concentrations by measuring absorbance at 595 nm. Top: graph shows absorbance values of the supernatant after nanoparticle formation. Bottom: cuvettes containing standard solutions (left) and the sample solution (right). Data from Table 3.

Comments

ddelphine: How did you calculate the 72% efficiency? Maybe explicitly write the calcul somewhere could be useful since the explanations were not clear in the Material and methods.

alvinwang: Thank you for your comment! We included a sample calculation in the method section.



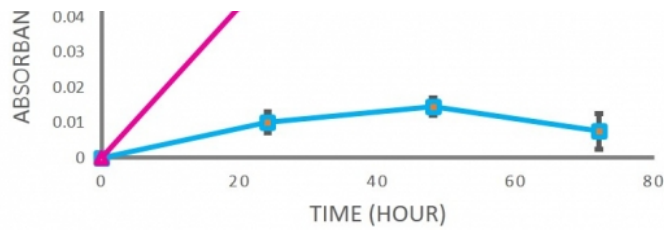


Figure 12. BSA proteins are released from chitosan nanoparticles at 37°C, but almost no change occurred at 4°C. Data from Table 4.

Comments

ddelphine: You should write again the you measured the absorbance at 595 nm after performing a Bradford assay on the solution containing your nanoparticles.
alvinwang: Thank you for your comment! We've added the wavelength measured.

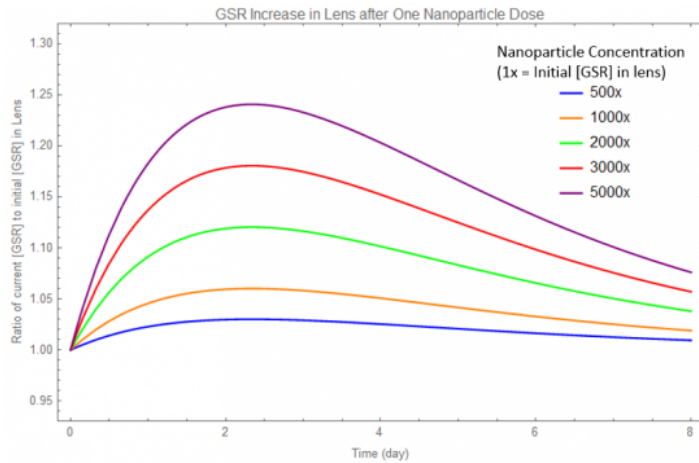


Figure 13. Single Dose of Nanoparticles Concentration Graph. Model of how GSR concentration in the lens is increased as a result of one dose of nanoparticles releasing GSR. Each curve represents a different concentration of GSR encapsulated in the nanoparticle. The GSR concentration in the lens increases, then decreases back to the initial, due to degradation.

Comments

ddelphine: I don't understand if it is the concentration of GSR inside one nanoparticle that increases or if it is the concentration of nanoparticle applied on the eye.
alvinwang: Thank you for your comment! In the graph, we set the initial concentration of GSR inside the nanoparticles. Then, the concentration inside the nanoparticles decrease as the concentration outside the nanoparticles, in the lens, increases. The different colors in the legend represent varying initial concentrations inside the nanoparticles. The curves in the graph, however, represent the changing concentrations outside of the nanoparticle, in the lens. This is why they increase, before falling back to equilibrium. We've update the legend in the graph to hopefully clarify this.

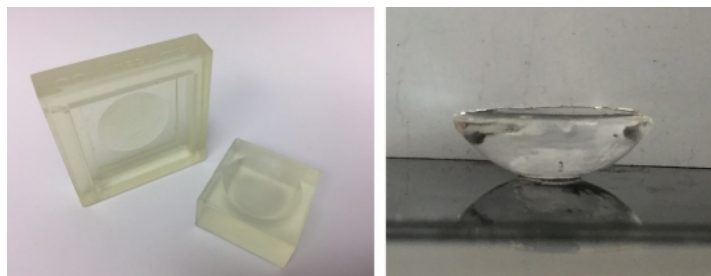
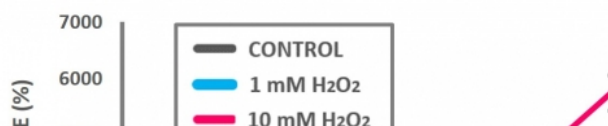
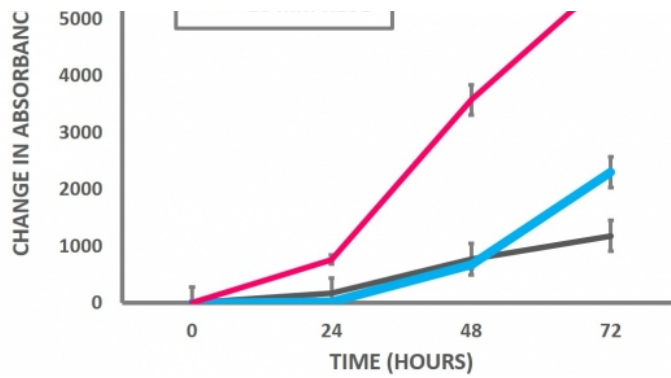


Figure 14. A 3D-printed mold (left) for making hydrogel contact lenses (right).

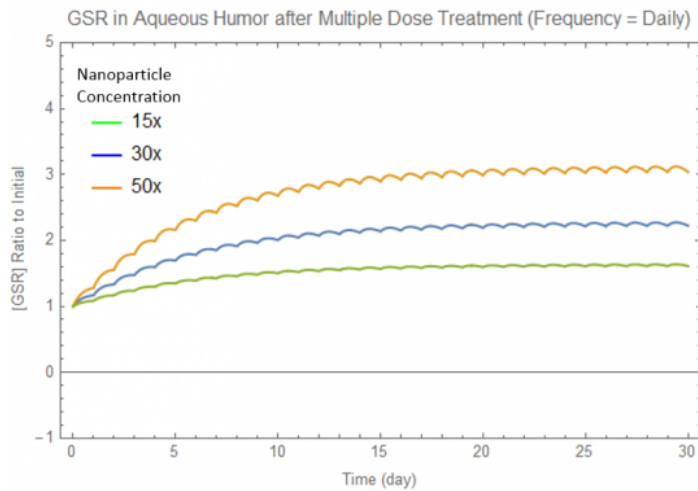




Comments

jejohnston: What was your control? Was it a negative control or a positive control? It would be good if you could mention the conditions in the figure legend.
ddelphine: You should write also in the legend the wavelength for the absorbance measurement.
alvinwang: Thank you for your comments! We've included that the control has no H₂O₂ and the wavelength measured.

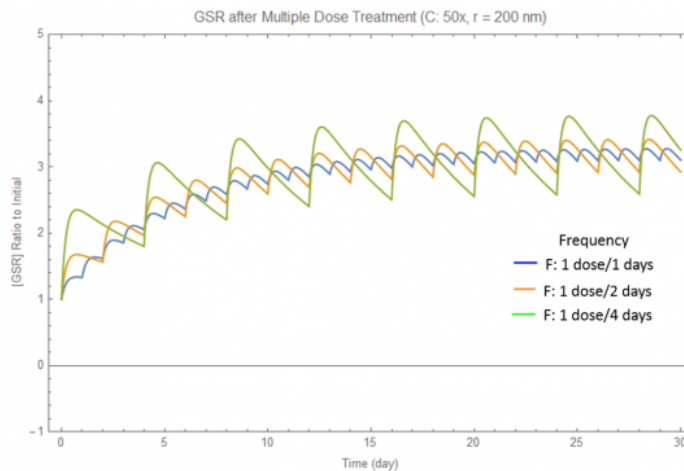
Figure 15. Increasing the amount of H₂O₂ leads to a greater increase in lens solution absorbance. Data from Table 5.



Comments

ddelphine: It would be better to scale the y axis and remove the negative part (that is completely useless here).
alvinwang: Thank you for your comment! We've update the y-axis.

Figure 16. Multiple Dose of Nanoparticles Concentration Graph. When patients are given GSR-loaded nanoparticles daily, the resulting change in GSR concentrations in their lens is shown. Each curve represents a different amount of nanoparticle concentration in the eye drop.



Comments

ddelphine: Same as for my comment for figure 16 on the scale of y-axis.
ddelphine: On the graph, your legend says 1 dose but from the legend below each dose doesn't contain the same concentration of nanoparticles so the concentration of

each dose should be clearly written.

alvinwang: Thank you for your comment! We have changed the y axis and updated the figure caption to say, "Over the same time period, we deliver the same amount of GSR. We can change the number of doses and the time between each dose to deliver this amount. We find that a low daily dose is preferable over occasional large doses, because the GSR concentration remains more stable (without sudden fluctuations)."

Figure 17. Changing Frequency. The equilibrium concentration is unaffected, although lower frequencies (greater periods between dose), with a higher concentration each time, results in an unstable graph.

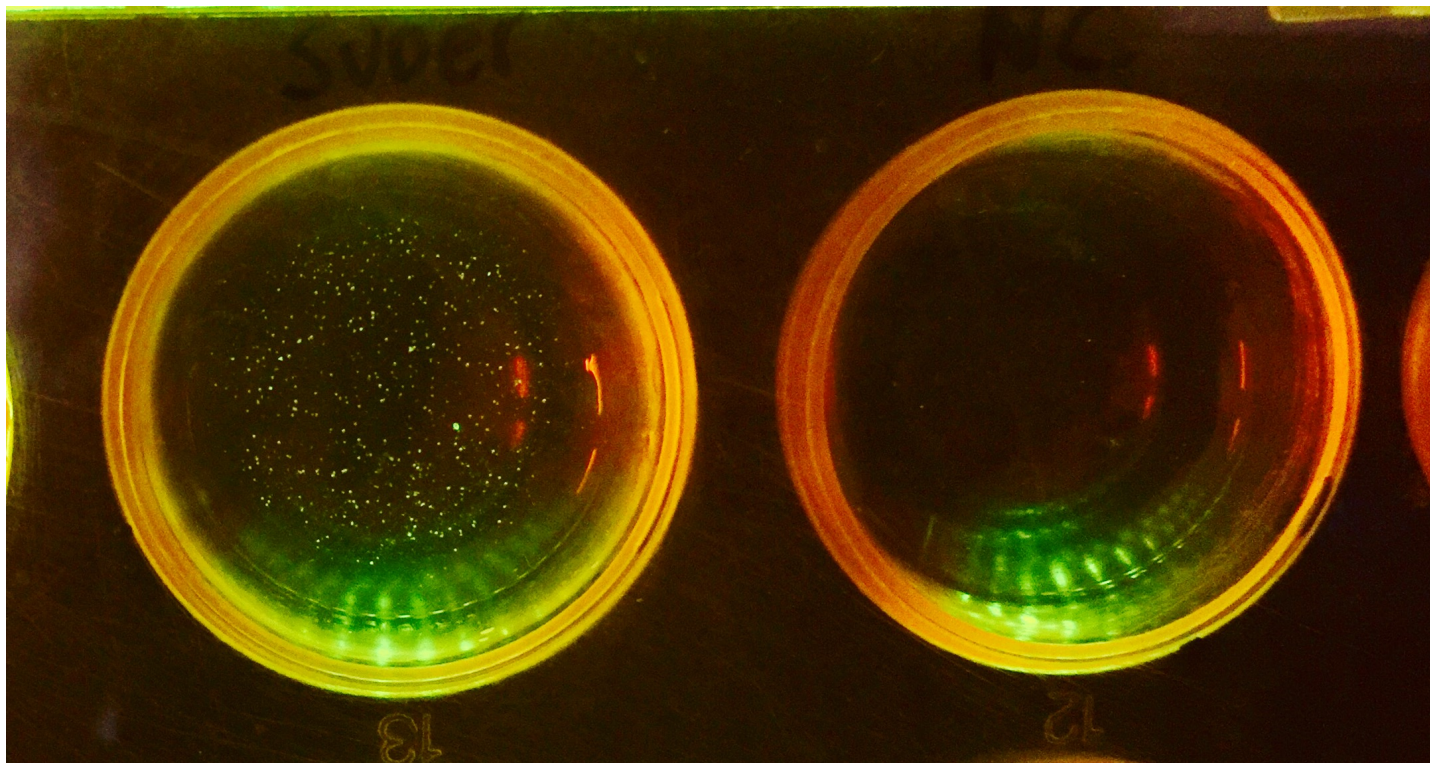


Figure 18. Contact lenses embedded with GFP-containing nanoparticles (left) and without GFP nanoparticles (right) in mold.

Table 1. Effect of GSH on preventing cataract development.

Values are absorbances measured at 397.5 nm.

Time (hours)	0 24	48	72
Control	0.165	0.770	1.180
10mM H ₂ O ₂	0.762.5	3.568.55	5.745.65
10mM H ₂ O ₂ + GSH	0.170.3	2.006.5	3.685

Table 2. Effect of 25HC on reversing cataract development.

Values are absorbances measured at 397.5 nm.

Time after H ₂ O ₂ addition (hours)	0 48	72
Control	0.2.734	9.193
10mM	0.489.515	1.581.977
10mM + Vet drops	0.864.607	1.331.71
10mM + 20uM 25hc	0.600.784	1.307.44
10mM + 50uM 25hc	0.353.747	744.9

Table 3. Protein encapsulation of chitosan nanoparticles.

Values are absorbances at 595 nm (Bradford)

Protein Concentration (mg/mL)	Absorbance (Trial 1)	Absorbance (Trial 2)
2	0.832	0.667
1.5	0.727	0.577
1	0.651	0.453
0.75	0.559	0.314
0.5	0.45	0.255
0.25	0.318	0.151
0	0.173	0
unknown	0.424	0.243

Table 4. Protein release of chitosan nanoparticles.

Values are absorbances at 595 nm (Bradford). Each temperature has 3 samples.

Temperature (celsius)	Time (hours)								
	24			48			72		
-20	0.008	0.002	0.005	0.017	0.013	0.015	0.006	0.009	0.0075
4	0.013	0.017	0.01	0.017	0.012	0.0145	0.008	0.007	0.0075
37	0.05	0.053	0.0515	0.056	0.055	0.0555	0.071	0.07	0.0705

Table 5. Effect of H₂O₂ on lens solution absorbance at different concentrations.

Values are absorbances measured at 397.5 nm.

Time (hours)	0	24	48	72
Control	0	165	770	1180
1mM H ₂ O ₂	0	18.2	668.4	2300
10mM H ₂ O ₂	0	762.5	3568.55	5745.65

Data tables.xlsx (/files/posts/4626945318317983602/a32ae76f121f9cfb5aa5e3d8082a7eaa_Data tables.xlsx)

Interpretation:

Cataract treatment is unavailable to many people in the world today, even though 20 million people suffer from cataracts and this number is expected to expand rapidly with the aging population (National Health Institute). Here we constructed plasmids expressing protein drugs and showed their potential to prevent and treat cataract. We then synthesized chitosan nanoparticles to deliver the proteins non-invasively. Since the materials for culturing bacteria and synthesizing chitosan nanoparticles are inexpensive compared to the cost of surgery, this system has the potential to serve as an alternative to surgery.

To show that glutathione reductase (GSR) and 25-hydroxylase (CH25H) could prevent and treat cataracts respectively, we tested their enzymatic products in a cataract model. We constructed the cataract model by extracting soluble proteins from fish lens and oxidizing the proteins with H₂O₂. After adding different concentrations of H₂O₂, our results show that increasing concentrations of H₂O₂ led to more severe cataracts (Figure 15). We also ran a protein gel to compare the sizes of untreated and H₂O₂-treated proteins. After treatment with H₂O₂, there was an increase in higher bands, which confirms that proteins were clumping and aggregating (Figure 2). Together, the protein gel and our absorbance data suggest that our experimental model accurately represents cataract development.

Comments

ddelphine: There also instead of "protein gel", you should say at least one time that it is a SDS-PAGE. You can also say gel electrophoresis even if it is less informative, it is more accurate that "protein gel".

alvinwang: Thank you for your comment! We've replaced the wording from "protein gel" to "SDS-PAGE".

Using the cataract model, we demonstrated that GSH and 25HC can prevent and treat cataract respectively. GSH-treated solutions showed lower absorbance values compared to the untreated control, indicating less protein aggregation (Figure 3). These results suggest that GSH has a preventative effect on cataract formation. 25HC was shown to reduce the absorbance of lens solution that already reacted with H₂O₂. In addition, a higher concentration of 25HC lowered the absorbance even further (50 μM compared to 20 μM). We compared 25HC to commercial pet eye drops, which did not lower the absorbance, suggesting that 25HC is more effective than the commercial product (Figure 4). Although our results demonstrate that 25HC can reverse cataract, researchers do not fully understand the mechanism. Since GSH and 25HC are produced by GSR and CH25H respectively, GSR and CH25H could be delivered to the lens as cataract prevention and treatment.

The cornea protects the eye from foreign materials, but it also prevents drugs from reaching the lens (Gaudana et al., 2010). Researchers have developed several methods to penetrate the cornea and deliver drugs to the lens, but many are invasive, such as implants (Patel et al., 2013). The most promising method is using chitosan nanoparticles as drug carriers (Cholkar et al., 2013). Researchers have used chitosan nanoparticles in the eye; their low toxicity to somatic cells makes them safe and they do not affect the anatomy of the eye (Enriquez de Salamanca et al., 2006). They can embed in the cornea, and their biodegradability allows the drug to be released continuously into the eye (Enriquez de Salamanca et al., 2006; Campos et al., 2005). We synthesized chitosan nanoparticles and proved that they encapsulated proteins with a high efficiency 72% (Figure 11). By measuring protein release at different temperatures, we showed that the nanoparticles would degrade at 37°C and release the desired drugs (Figure 12). We also measured minimal protein release at 4°C. This finding suggests that we can store a final functional product (e.g., eye drop) at 4°C without nanoparticle degradation, while the proteins can be released from nanoparticles when the eye drop is applied at body temperature.

Our long-term nanoparticle models show that GSR concentration in the eye increases as expected. However, the GSR concentration approaches a limit after about 3 weeks, and further eye drop use will maintain the GSR concentration. This limit is proportional to the concentration of GSR in the nanoparticles (Figure 16). Therefore, we desire that the GSR concentration to equal this equilibrium concentration. Also, it is better to apply small concentrations of nanoparticles frequently as opposed to larger concentrations occasionally (Figure 17), as we want to maximize the stability of GSR concentrations within the eye. These suggestions are valuable to manufacturers and clinics who may utilize these eye drops. We also considered the application of proteins using nanoparticle-embedded contact lens, which would continuously deliver proteins if worn by the patient (Figure 18). Non-invasive delivery of protein drugs to the lens is a promising approach to prevent and treat cataracts.

References:

Comments

jjoupil: Nice list of references! I believe PLOS uses "Vancouver" style; if you would like to publish your article, you will need to reformat this list and change your in-text citations.

alvinwang: Thank you for your comment! We've changed our reference style to Vancouver.

Bio-Rad. (n.d.). Quick Start™ Bradford Protein Assay [PDF]. Hercules: Bio-Rad Laboratories, Inc..

Behl, G., Iqbal, J., O'Reilly, N. J., McLoughlin, P., & Fitzhenry, L. (2016). Synthesis and Characterization of Poly (2-hydroxyethylmethacrylate) Contact Lenses Containing Chitosan Nanoparticles as an Ocular Delivery System for Dexamethasone Sodium Phosphate. *Pharmaceutical research*, 1-11.

Campos, A. M., Diebold, Y., Carbalho, E. L., Sánchez, A., & Alonso, M. J. (2005). Chitosan Nanoparticles as New Ocular Drug Delivery Systems: In Vitro Stability, in Vivo Fate, and Cellular Toxicity. *Pharm Res Pharmaceutical Research*, 22(6), 1007-1007. doi:10.1007/s11095-005-4596-x

Cvekí, A. & Ashery-Padan, B. (2014). The cellular and molecular mechanisms of vertebrate lens development. *Development*, 141(23), 4432-4447

Ovchik, A., & Astley-Flaud, R. (2014). The cellular and molecular mechanisms of vertebrate lens development. *Development*, 141(29), 4432-4447.

Cholkar, K., Patel, S. P., Vadlapudi, A. D., & Mitra, A. K. (2013). Novel Strategies for Anterior Segment Ocular Drug Delivery. *Journal of Ocular Pharmacology and Therapeutics*, 29(2), 106-123.

Enriquez De Salamanca, A., Diebold, Y., Calonge, M., García-Vazquez, C., Callejo, S., Vila, A., & Alonso, M. J. (2006). Chitosan nanoparticles as a potential drug delivery system for the ocular surface: toxicity, uptake mechanism and in vivo tolerance. *Investigative ophthalmology & visual science*, 47(4), 1416-1425.

Fraunfelder, F.T., Fraunfelder F.W., & Chambers, W.A. (2008) *Clinical Ocular Toxicology: Drug-Induced Ocular Side Effects*. Saunders

Gan, Quan, and Tao Wang. "Chitosan nanoparticle as protein delivery carrier—systematic examination of fabrication conditions for efficient loading and release." *Colloids and Surfaces B: Biointerfaces* 59.1 (2007): 24-34.

Gaudana, R., Ananthula, H.K., Parenky, A., Mitra, A.K. (2010). Ocular Drug Delivery. *AAPS Journals*, 12(3), 348-360 doi: 10.1208/s12248-010-9183-3

Giblin, F. J. (2000). Glutathione: a vital lens antioxidant. *Journal of Ocular Pharmacology and Therapeutics*, 16(2), 121-135.

Giorgio, M., Trinei, M., Migliaccio, E., & Pelicci, P. (2007). *Nature Reviews Molecular Cell Biology*, 8(9), 722-8.

Makley, L. N., McMenimen, K. A., DeVree, B. T., Goldman, J. W., McGlasson, B. N., Rajagopal, P., Duniyak, B.M., McQuade, T.J., Thompson, A.D., Sunahara, R., Klevit, R.E., Andley, U.P., and Gestwicki, J.E. (2015). Pharmacological chaperone for α -crystallin partially restores transparency in cataract models. *Science*, 350(6261), 674-677.

Mello, C. M., Arcidiacono, S., Garvey, M., Gerrard, J., Healy, J., Soares, J., ... & Wong, K. (2012). Identification of Important Process Variables for Fiber Spinning of Protein Michael, R., & Bron, A. J. (2011). The ageing lens and cataract: a model of normal and pathological ageing. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 366(1568), 1278-1292.

National Eye Institute | Cataracts. (n.d.). Retrieved October 04, 2016, from <https://nei.nih.gov/eyedata/cataract> (<https://nei.nih.gov/eyedata/cataract>)

Patel, A., Cholkar, K., Agrahari, V., & Mitra, A. K. (2013). Ocular drug delivery systems: an overview. *World journal of pharmacology*, 2(2), 47.

Segre L (2016, Sept. 21). Cataract surgery cost. Retrieved from <http://www.allaboutvision.com/conditions/cataract-surgery-cost.htm> (<http://www.allaboutvision.com/conditions/cataract-surgery-cost.htm>)

Truscott, RJ (2005). Age-related nuclear cataract-oxidation is the key. *Exp Eye Res.*, 80(5): 709-25.

Wilson, T., Aeschlimann, R., Tosatti, S., & Lorenz, K. E. (2014). Defining 'Fresh' Corneal Tissue for Utilization in Determining Human Cornea Coefficient of Friction Values. *Investigative Ophthalmology & Visual Science*, 55(13), 1506-1506.

World Health Organization | Priority eye diseases. (n.d.). Retrieved October 03, 2016, from <http://www.who.int/blindness/c...> (<http://www.who.int/blindness/causes/priority/en/index1.html>)

Comments

Paul: Hello, My name is Paul, I'm the Deputy Editor of PLOS Medicine. I enjoyed reading this article, cataracts are an important health problem and I think it's a neglected topic given the scale of the problem. I enjoyed reading this manuscript. If I was the editor for this manuscript I'd be looking for reviewers to give me and the authors some advice on how to improve the presentation of this manuscript and I think there are two big picture points peer-reviewers could consider here: 1) How could the authors improve the clarity of their presentation for engaged but non-expert readers? Here's two examples: * It might be useful in the title and abstract to say that the study uses a fish lens experimental model and mathematical modelling. * "We also added 3 drops of pet eye drops for comparison." What's the active ingredient in the OcluVet pet eye drops and what is the concentration? Does this matter so that we readers can interpret Figure 4? 2) Does the interpretation of the current version of the manuscript overreach the data that is presented? Here's an example: "...we demonstrated that GSH and 25HC can prevent and treat cataract respectively" Is this the case? If not what could be written to reflect the data more precisely? What are the next set of questions that need to be addressed in order to achieve the end goals – prevention and treatment of cataracts? I'm looking forward to seeing the feedback from reviewers! All the best Paul

alvinwang: Dear Paul, We have made changes in the manuscript to better reflect the results. For example, in the abstract, we added, "we simulated cataract formation in fish lens proteins using hydrogen peroxide, and in this model we find that both glutathione and 25-hydroxycholesterol effectively decrease the cloudiness of fish lens solutions." We have tried to make it clearer which experiments were performed using fish lens proteins as a cataract model, and that at the end, mathematical modelling was used to simulate nanoparticle and GSR release. Throughout the text, we have also tried to more accurately describe the results (using phrases like "decrease opacity" or "decrease absorbance" instead of just saying "treat cataracts." The commercial eye drop, OcluVet, was included as a comparison group to our proposed treatment, and we have added the active ingredients and suggested daily dosage in the methods part. We have also added a paragraph on future work that discusses the next steps, such as testing whether we can actually purify and encapsulate our target proteins, GSR and CH25H. We hope these changes have made the manuscript more complete! Alvin

PLOSFeedback: Hi @alvinwang (/user/alvinwang) Thanks for your submission to the PLOS iGEM Project. Our editorial team have reviewed your article and have the following feedback: The idea of the study is interesting, with the authors trying to cure cataracts with a specific molecule as an alternative to surgery. However, the reporting could do with more detail to clarify exactly what was done – it is unclear if human 'patients' were used, or if Figure 16 is based on modelling data. Most of the reporting seems to be based on in vitro data, so it is quite preliminary.

alvinwang: Thank you for your comments! We've updated the text to show that most of the experiments were performed using fish lens proteins. We have also clarified, in the revised version, that figures 14-16 reflect modelling data.

tsihavong: Hi, I'm a member of the Stanford-Brown iGEM team! My feedback is that it might be useful to include how a fish lens model is representative of a human lens, and how your data can be cross-applied when dealing with human cornea. Particularly with your modeling, I want to be shown how the Noyes-Whitney equation is applicable and why it made your model accurate. I think it would also be useful to have a sort of "next step" section.

alvinwang: Thank you for your comment! We've added that we chose to use fish lens in our cataract model because fish was readily available to us and because fish lens contain similar crystallin proteins as humans do. For modelling, the drug has to diffuse through the chitosan nanoparticle. However, we don't know at what rate this diffusion occurs, so we referenced Fraunfelder et al. 2008. The paper describes multiple methods of modeling nanoparticle drug diffusion, and concluded that the Noyes Whitney equation was most inclusive of different types of nanoparticle diffusion. Initially, we didn't know the thickness of the diffusion layer, so we estimated the value using literature data, and then created a model for nanoparticle diffusion. Then we compared the model to our nanoparticle release data, and corrected the value of the diffusion layer. We've also added a discussion of future steps in our revised manuscript.

ddelphine: Hello, I am a member of the Evry 2016 iGEM team. First, I wanted to say that I found your study really interesting and compliment your work on the subject. I have a few comments. There are maybe too much tables. I suggest that you keep the most important ones and keep the other for supplementary data or something. You should also think about alternate the figures and tables and thus, put the tables related to the curves just after them. It would be clearer like that. Then, I have a few questions about your work. You use UVs to synthesize your contact lenses. Did you think about their impact on the protein activity? UVs can denature proteins so even if they are encapsulated inside your nanoparticles, I think that there is a risk of losing their activities. On the same idea, you could discuss about the stability of your product and the conservation time. You determined that at 4°C the proteins were not released from the nanoparticles but enzymes don't keep their activities very long,

even when stored at 4°C, and it is enzymes dependent. So, maybe your nanoparticles will still contain your enzymes but they will not be active anymore. I think you could add some little comments on that in the discussion. Overall it was really a good work so thank you for sharing your results here today!

alvinwang: Dear Delphine, thank you for your comments! We've moved some of our data to the supplement. We've also added a discussion of our future work, including testing the enzymatic activity of GSR and CH25H after storage and UV exposure.

alvinwang: Thanks very much for sharing your comments and the opportunity to share our work! We will revise our report to answer all your questions and very insightful feedback.