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Age-dependency of molecular diffusion in the human anterior lens capsule assessed using fluorescence recovery after photobleaching

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Purpose: To quantify the partition coefficient and the diffusion coefficient of metal-carrier proteins in the human lens capsule as a function of age.

Methods: Whole lenses from human donors were incubated overnight in a solution of fluorescently labeled transferrin, albumin, or ceruloplasmin. In the central plane of the capsule thickness, fluorescence recovery after photobleaching (FRAP) experiments were conducted to measure the diffusion of the protein within the lens capsule. The anterior portion of the lens was recorded before the FRAP experiments to locate the boundaries of the anterior lens capsule and to measure the partition coefficient of the labeled proteins. The partition coefficient (P), the time to half maximum recovery of the fluorescent intensity ($\tau_{1/2}$), and the diffusion coefficient (D) for each protein were analyzed as a function of donor age.

Results: There was no statistically significant relationship between the half maximum recovery time or the diffusion coefficient and age for transferrin (molecular weight [MW]=79.5 kDa, $\tau_{1/2}$=17.26±4.840 s, D=0.17±0.05 μm^2/s), serum albumin (MW=66.5 kDa, $\tau_{1/2}$=18.45±6.110 s, D=0.17±0.06 μm^2/s), or ceruloplasmin (MW=120 kDa, $\tau_{1/2}$=36.57±5.660 s, D=0.08±0.01 μm^2/s). As expected, the larger protein (ceruloplasmin) took longer to recover fluorescent intensity due to its slower movement within the lens capsule. The partition coefficient statistically significantly increased with age for each protein ($P_{albumin}$: 0.09–0.71, $P_{ceruloplasmin}$: 0.42–0.95, $P_{transferrin}$: 0.19–1.17).

Conclusions: The diffusion of heavy-metal protein carriers within the anterior lens capsule is not dependent on age, but it is dependent on the size of the protein. The permeability of the lens capsule to these heavy-metal protein carriers increases with age, suggesting that there will be a higher concentration of heavy metals in the older lens. This behavior may favor the formation of cataract, because heavy metals enhance protein oxidation through the Fenton reaction.

The lens capsule is a basement membrane encapsulating the lens of the eye. It is a semipermeable membrane allowing molecules important for lens physiology to enter, while protecting it from harmful agents [1,2]. As no evidence has been found for pores in the capsule, molecules important for lens biology must passively diffuse through the interwoven network of the capsule to reach the avascular lens. Early studies of lens capsule permeability using diffusion chambers have shown that the lens capsule is permeable to water [3-5], electrolytes [6-9], simple sugars [3,4,7,10], amino acids [11,12], and larger macromolecules (including α, β, and γ crystallins [13] and albumin [14,15]). Research has also shown that the permeability of the lens capsule decreases with increasing molecular weight [2,3,10,16,17], although molecular weight alone cannot be used to determine capsule permeability [2].

The human lens capsule is known to undergo several modifications with age, including an increase in thickness [18,19] and a decrease in elasticity [18,20-23]. Recent studies have also shown variations in surface structure and roughness with age [24-26]; it is possible that similar changes exist throughout the depth of the capsule. Changes in thickness, elasticity, and structure could result in changes to the lens capsule permeability with age. Early studies using calf and cow lenses found that lens capsule permeability to simple sugars, including glucose, statistically significantly decreased with age [3,4]. Because the lens is avascular, it must rely on the ability of the lens capsule to allow entry of molecules important for overall lens function. Therefore, modifications to transport through the capsule with age would have a negative impact on lens physiology.

The purpose of the present study was to build upon previous investigations and quantify the diffusion of multiple proteins within and through the human lens capsule. The specific proteins selected, transferrin, serum albumin, and ceruloplasmin, are known carriers for heavy metal ions, such as Fe^{3+} and Cu^{2+} [27-32]. Although the proteins are found in the aqueous humor [33], the metal ions are known to exacerbate oxidative stress via Fenton reaction catalysis. The presence of these ions is suspected to play a role in cataract formation

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[34,35]. An innovative method using confocal microscopy and recent advances in photobleaching techniques was used to quantify diffusion within the extracellular matrix of the lens capsule. This technique has been previously applied to quantify diffusion in the mouse lens capsule [2].

METHODS

Tissue preparation protocol: Experiments were conducted on 34 lenses from 20 human cadavers (ages 20–69 years) provided by the Florida Lions Eye Bank (Table 1). When the technicians harvest corneas suitable for transplantation, they also remove the lenses specifically for our laboratory. The lenses are extracted from donors immediately post-corneal harvesting for transplantation and placed in sealed vials containing Dulbecco's modified eagle medium (DMEM, D1145, Sigma-Aldrich, St. Louis, MO) until they can be further prepared for imaging. The media were not changed during this storage period; therefore, we did not control for lens epithelial cell viability. All human lenses were obtained and used in compliance with the guidelines of the Declaration of Helsinki for research involving the use of human tissue. Because preliminary experiments demonstrated that the data were inconsistent for samples with postmortem times greater than 9 days, only samples 9 days postmortem and less were included in this study (5.6±2.0 days, range: 2–9 days).

The day before experimentation, whole lenses were placed in a solution of fluorescently labeled transferrin (n=12), albumin (n=11), or ceruloplasmin (n=11) overnight. Transferrin is the carrier for iron (Fe) [30,31], ceruloplasmin transports copper (Cu) [32], and serum albumin binds cadmium (Cd) [36], copper [27-29], and zinc (Zn) [29]. The base solution for each of these proteins was DMEM, to minimize delamination of the lens capsule during the staining period [37]. Transferrin was conjugated with fluorescein (Thermo Fisher Scientific, Waltham, MA, T2871), serum albumin was conjugated with Alexa Fluor™ 488 (Thermo Fisher Scientific, A13100), and ceruloplasmin (Millipore Sigma, Burlington, MA, 239799) was conjugated with fluorescein-5-EX (Thermo Fisher Scientific, F10240). Paired eyes were divided between two different proteins to maintain statistical independence of each data set.

Fluorescence recovery after photobleaching: Fluorescence recovery after photobleaching (FRAP) was used to characterize molecular diffusion in the human lens capsule. Because the thickness of the human anterior lens capsule ranges between 20 and 25 µm, FRAP measurements were centralized in a 10 µm thickness window situated 5–10 µm from the anterior surface of the lens capsule. The measurement location was established by imaging a three-dimensional z-stack of the anterior capsule preceding the FRAP experimentation (Figure 1A). When the three-dimensional z-stacks were conducted, the 405 nm and 488 nm laser lines were used. The autofluorescence of the lens capsule at 405 nm revealed the boundaries of the capsule, and assisted in determining the proper FRAP measurement location. Once the measurement plane in the z-direction was established, a single image was acquired to establish the baseline fluorescent intensities. The area to be bleached was then selected in the center of the field of view (FOV; Figure 1B). This stimulated region of interest (ROI), a circle of 20 µm diameter, was bleached for a total of 2 s using an argon laser (488 nm, pinhole 1.2 Airy units, beam diameter 25.5 µm) set to full power (65 mW). Immediately post-bleaching, time-lapse images of the full FOV were captured each second for at least 3 min (Figure 1C). As the amount of fluorescent intensity decreases as a result of laser exposure during time-lapse imaging, a reference ROI of 15 µm diameter, situated at the periphery of the FOV, was designated to compensate for continuous photobleaching. Changes in fluorescence intensity stemming from the diffusion of the fluorescently labeled molecule were monitored in a standard region (diameter: 7 µm) within the bleached area (Figure 1D). These experiments were repeated in at least three different planes along the z-axis of the capsule within 10 µm of the central depth. Measurements were also repeated at two different regions of interest within each plane.

The analysis software included with the Nikon A1R confocal microscope was used to determine the time point at which 50% of the fluorescent intensity of the photobleaching had been restored ("half maximum recovery time"). Because the values obtained along the central 10 µm were not statistically significantly different from each other, all values obtained at the different locations were averaged for the final value for that sample. Assuming isotropy, the diffusion coefficient was calculated from this value, using the Soumpasis equation [38]:

$$D=0.224\frac{r^2}{\tau_{\frac{1}{2}}}$$

where r is the radius of the analyzed area (3.5 µm in these experiments), and $\tau_{1/2}$ is the half maximum recovery time that is provided by the confocal microscope software. All data were analyzed as a function of donor age using linear regression.

Before each FRAP experiment was performed, a cross-sectional z stack of the lens capsule was acquired. We noticed a high fluorescent intensity between the coverslip and the lens capsule, corresponding to the fluorescent dye within the

Table 1. Summary of donor age, postmortem time, half maximum recovery time, diffusion coefficient, and partition coefficient of all samples included in this study.

	Age (years)	Postmortem Time (days)	Half Maximum Recovery Time (s)	Diffusion Coefficient (μm²/s)	Partition Coefficient
ALBUMIN					
	20	4	26.89	0.102	0.09
	30	7	16.39	0.167	0.33
	31	8	27.75	0.099	0.16
	43	7	16.38	0.168	0.2
	46	4	9.03	0.304	0.28
	52	3	17.35	0.158	0.21
	53	9	15.95	0.172	0.37
	57	5	10.49	0.262	0.33
	60	6	24.84	0.11	0.54
	65	4	17.22	0.159	0.27
	65	6	20.65	0.133	0.71
CERULOPLASMIN					
	24	7	39.45	0.07	0.42
	40	5	31.72	0.087	0.46
	48	7	39.15	0.07	0.51
	53	4	29.1	0.094	0.55
	55	3	37.46	0.073	0.51
	56	6	33.25	0.083	0.53
	62	7	47.36	0.058	0.95
	63	3	35.11	0.079	0.9
TRANSFERRIN					
	20	4	13.65	0.201	0.19
	31	9	11.09	0.247	0.61
	43	4	19.4	0.141	0.62
	46	9	11.77	0.233	0.35
	48	7	21.5	0.128	0.38
	52	6	15.05	0.182	0.25
	55	3	26.83	0.102	0.96
	57	7	16.95	0.162	1.17
	60	7	15.39	0.178	0.82
	62	7	19.42	0.141	0.84
	65	4	22.91	0.12	0.68
	65	9	13.11	0.209	0.75

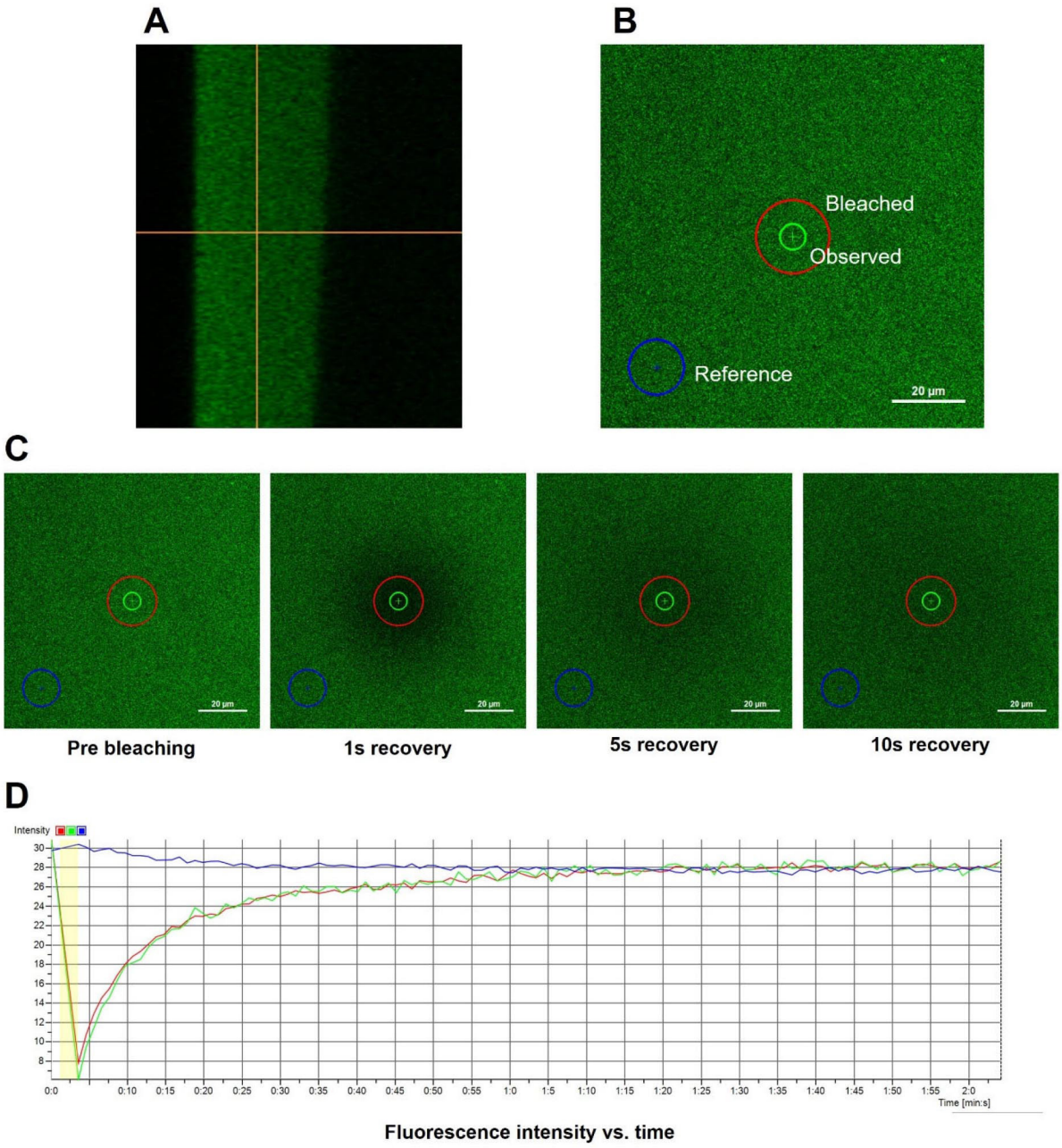

Figure 1. FRAP protocol. **A**: Confocal image of a three-dimensional (3D) z-stack of the anterior capsule used to locate an area within the sample. Crosshairs indicate the starting location of the fluorescence recovery after photobleaching (FRAP) protocol. **B**: Designated regions of interest (ROIs). Bleached ROI, red circle of 20 µm diameter, is the area to be bleached. Reference ROI, blue circle of 15 µm diameter, is identified for photobleaching correction. Observed ROI, green circle of 7 µm diameter, is the area monitored for fluorescence recovery. **C**: Time-lapse images of the full field of view during the FRAP protocol. **D**: Quantitative monitoring of the fluorescence intensity within the ROIs. The time to half maximum recovery is indicated.

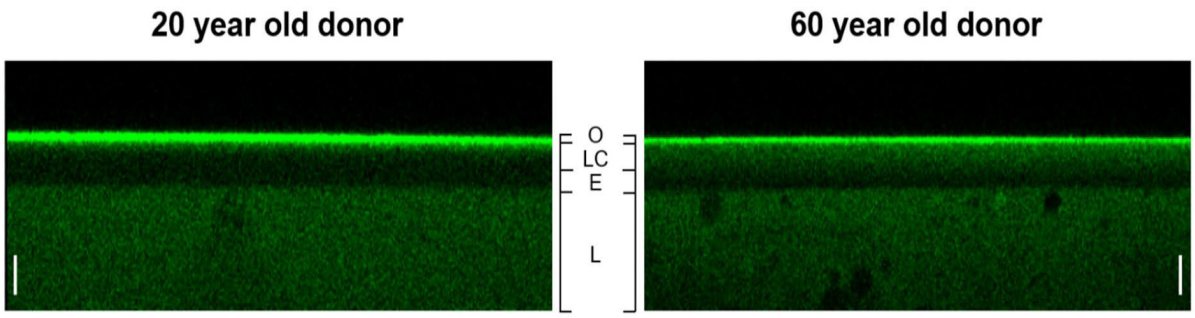

Figure 2. Confocal microscopy cross-sectional images showing a bright band of fluorescently labeled transferrin outside the lens capsule from a young donor (left); in contrast, fluorescently labeled transferrin is seen inside the lens capsule from an older donor (right). O: outside the lens capsule, LC: inside the lens capsule, E: epithelial cells, L: lens. The scale bar represents 10 µm.

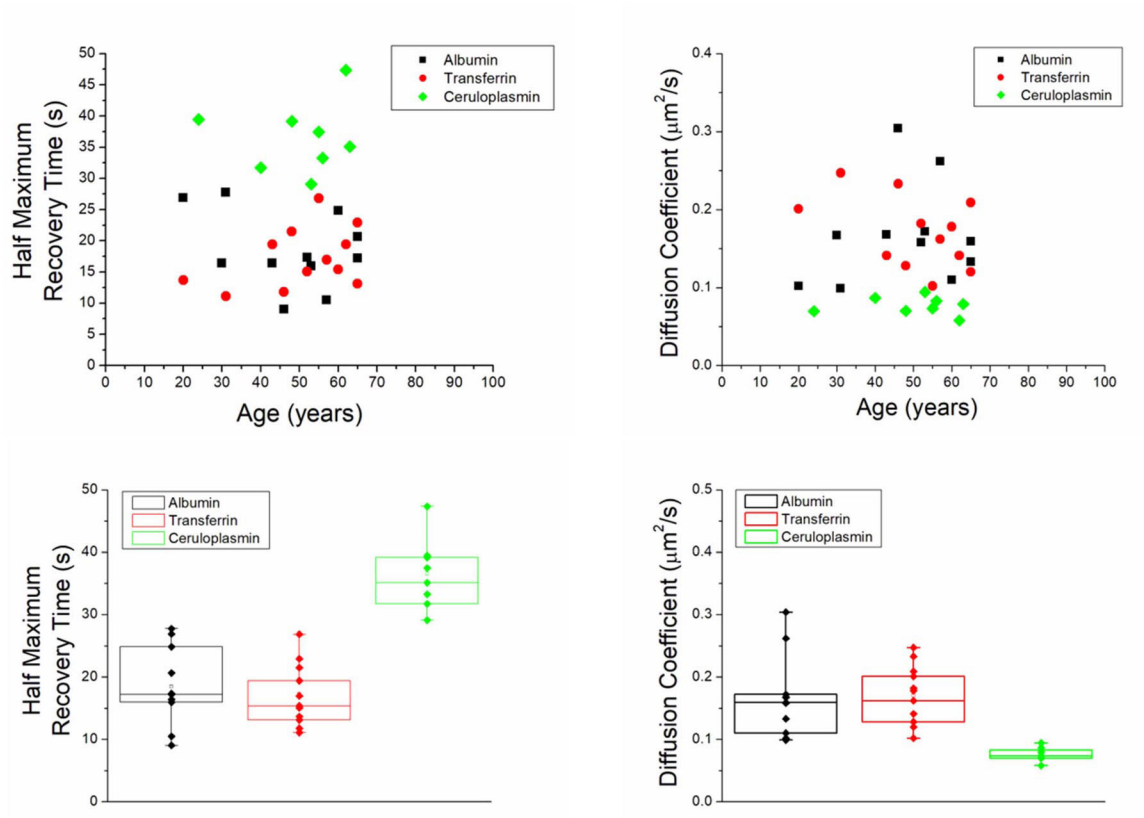

Figure 3. Half maximum recovery time and diffusion coefficient measured within the anterior lens capsule for all samples. Ceruloplasmin movement was statistically significantly slower than albumin and transferrin.

fluid (Figure 2). We took the ratio of the fluorescent intensity inside the lens capsule ($I_{capsule}$) to this fluorescent intensity outside the lens capsule (I_{media}) to calculate the partition coefficient, P:

$$P = \frac{I_{lens\ capsule}}{I_{media}}$$

The values used were from the first z-stack image obtained when the sample was placed in the confocal microscope. The partition coefficient for each protein was analyzed as a function of donor age.

RESULTS

Transferrin: FRAP experiments using fluorescein-labeled transferrin were successfully conducted on 12 human anterior lens capsules from 12 human donors (ages 20–69 years; Table 1). There was no statistically significant relationship between the half maximum recovery time and age (p=0.197) or the diffusion coefficient and age (p=0.139). The time to half maximum recovery of the fluorescent intensity was 17.26±4.840 s (range: 11.09–26.83 s), corresponding to a diffusion coefficient of 0.17±0.05 μm²/s (range: 0.10–0.25 μm²/s; Figure 3).

The ratio of the fluorescent intensity inside the lens capsule to the fluorescent intensity outside the lens capsule (partition coefficient) was 0.64±0.30 (range: 0.19–1.17). This ratio statistically significantly increases with age (p=0.042), which may indicate that the lens capsule blocks transferrin from entering the lens in the younger eye (Figure 4). The ratio reaches approximately 1 in the lens capsule from the older eye, which means that transferrin is freely passing into the lens capsule.

Albumin: FRAP experiments using Alexa Fluor™ 488 labeled albumin were successfully conducted on 11 human anterior lens capsules from 11 human donors (ages 20–69 years; Table 1). There was no statistically significant relationship between the half maximum recovery time and age (p=0.289) or the diffusion coefficient and age (p=0.512). The time to half maximum recovery of the fluorescent intensity was 18.45±6.110 s (range: 9.03–27.75s), corresponding to a diffusion coefficient of 0.17±0.06 μm²/s (range: 0.10–0.30 μm²/s; Figure 3).

The ratio of the fluorescent intensity inside the lens capsule to the fluorescent intensity outside the lens capsule (partition coefficient) was 0.32±0.18 (range: 0.09–0.71). This ratio statistically significantly increases with age (p=0.023), which may indicate that the lens capsule blocks albumin from entering the anterior lens capsule in the younger eye, whereas albumin flows freely inside the anterior lens capsule in the older eye (Figure 4).

Ceruloplasmin: FRAP experiments using fluorescein-labeled ceruloplasmin were successfully conducted on 11 human anterior lens capsules from eight human donors (ages 24–63 years; Table 1). Data from paired eyes were averaged to produce one data point per human donor to avoid skewing the data during statistical analysis. There was no statistically significant relationship between the half maximum recovery time and age (p=0.850) or the diffusion coefficient and age (p=0.952). The time to half maximum recovery of the fluorescent intensity was 36.57±5.660 s (range: 29.10–47.36s), corresponding to a diffusion coefficient of 0.08±0.01 μm²/s (range: 0.06–0.09 μm²/s; Figure 3).

The ratio of the fluorescent intensity inside the lens capsule to the fluorescent intensity outside the lens capsule

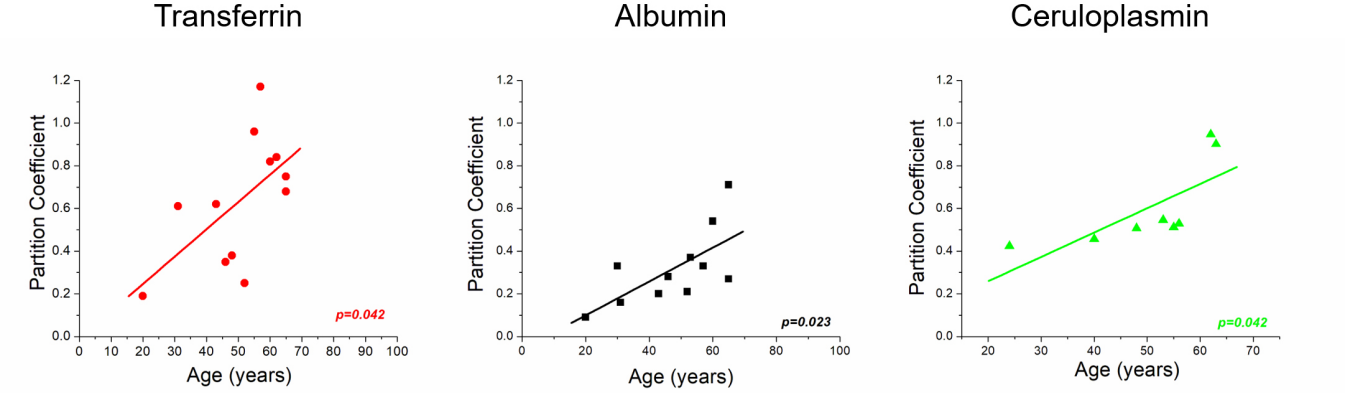

Figure 4. For all three proteins investigated, the partition coefficient statistically significantly increases with age. This suggests that the anterior lens capsule of the young lens is impermeable to heavy metal carriers, but that the anterior lens capsule of the old lens is fully permeable to heavy metal carriers.

(partition coefficient) was 0.60±0.20 (range: 0.42–0.95). This ratio statistically significantly increases with age (p=0.042; Figure 4). Therefore, as with albumin and transferrin, the anterior capsule in the younger eye blocks passage of ceruloplasmin, but the anterior capsule of the older eye allows it to freely flow inside.

DISCUSSION

Because the lens capsule serves as a semipermeable membrane controlling diffusion into the inner lens material, its permeability is of great interest. In this study, we quantified the age-dependency of protein diffusion into and within the central anterior lens capsule. We selected three proteins (transferrin, albumin, and ceruloplasmin) that are found in the aqueous humor. These three proteins are known carriers of heavy metals. Previous research has demonstrated that heavy metal concentration within the lens increases with age [39-41]. Although this increased heavy metal concentration could be due to accumulation within the lens with age, it could also be due to an increase in the diffusion of the protein carriers through the lens capsule. Increased multivalent metal concentration within the lens is thought to increase protein oxidation via the Fenton reaction, and could be a causative factor in cataract formation.

The half maximum recovery time and diffusion coefficients measured are indicative of movement within the XY plane of the lens capsule. When the FRAP experiments are run, the primary source of available fluorophores for recovery is from the XY plane. When the bleached, or stimulated, ROI is exposed to the laser at full power, the light path runs through the whole depth of the sample. At the set focal plane, the ROI will be in focus and bleached due to the full power of the laser; however, each plane above and below the focal plane will also be exposed to a high laser power in the general region set by the ROI. Because a considerable percentage of the proximal fluorophores in the Z plane will be bleached, the primary axis of transport that we are measuring would be radially, or in the XY plane. The data collected supported this hypothesis. When the FRAP experiments were performed, half maximum recovery times were collected for the whole bleached ROI (20 µm diameter), as well as for a smaller, standard ROI (7 µm diameter) centered within the bleached area. These measured times consistently showed a slightly faster recovery time for the stimulated, or bleached, ROI versus the recovery for the smaller, standard ROI. If the fluorophores contributing to the recovery of the bleached ROI were predominately due to Z axis transport, then the recovery times for both ROIs should be the same. The larger ROI recovered faster than the smaller ROI, which means that the transport of markers is due to radial transport in the XY plane.

We did not find any relationship between the time to half maximum recovery or the diffusion coefficient and age for any of the three proteins. These values were quantified in a central region of the anterior lens capsule at a depth approximately equal to the central plane. Therefore, these values are indicative of protein mobility once inside the capsule. The results suggested that proteins inside the capsule can move freely radially along the XY plane. The measurements were obtained at a plane at a depth corresponding to approximately 50% of the thickness; therefore, it is unknown whether this behavior would remain the same at different planes along the Z axis within the lens capsule. Given that each incremental depth of the capsule is a different layer, due to the capsule's thickness being deposited over the lifetime of the individual, as one progresses deeper into the thickness of the capsule, the age of each layer and its composition change. The outermost, or shallowest, layers of the lens capsule are the oldest, and have been exposed to the most oxidative stress. It would stand to reason that there may be a gradation within the entirety of the capsule depth, and any interaction with a protein possessing functional groups may differ as it traverses into the lens. It may also suggest that the age-related structural differences we measured using AFM at the surface [24,26] are not indicative of the structure deeper down in the depth of the lens capsule where the diffusion experiments were performed, and no age-related trends were observed. It would be interesting to repeat these measurements at different depths within the anterior lens capsule.

The analysis of the partition coefficient as a function of age provided an interesting relationship: For all three proteins, the partition coefficient increases statistically significantly with age. The partition coefficient gives a representation of lens capsule permeability to the proteins: A value of 0 means that the lens capsule is impermeable to that molecule (all outside compared to inside), and a value of 1 means that lens capsule is completely permeable to that molecule (the same amount outside and inside). Based on these results, the anterior lens capsule becomes more permeable to these heavy-metal carriers with increasing age. At a young age, the anterior lens capsule blocks the entry of the proteins into the lens. However, by approximately 50–60 years of age, the anterior lens capsule is freely permeable to these proteins. This finding may have important implications for cataract formation. Lens capsule permeability to heavy-metal carriers indicates that more heavy metals will enter the lens with age. The presence of heavy metals in the lens may enhance protein oxidation through the Fenton reaction. It is also possible that

the partition coefficients reflect the protein's affinity for the capsule extracellular matrix rather than its movement through it. This interpretation would be in agreement with the present results showing that the half maximum recovery time and the diffusion coefficient within the lens capsule do not change with age.

It would be expected that the mobility of the proteins within the lens capsule, as quantified by the time to half maximum recovery, would be dependent on the size of the proteins. In fact, previous studies performed by our group and others have found that the recovery time of larger molecular weight Dextran is longer than that of smaller molecular weight Dextran [2,17,42]. However, the recovery rate of the larger protein transferrin (79.5 kDa MW) was faster than the smaller protein albumin (66.5 kDa MW): 17.26±4.840 s for transferrin compared to 18.45±6.110 s for albumin. However, ceruloplasmin (120 kDa MW) is approximately twice the size of albumin, and the recovery time is approximately two times slower at 36.57±5.660 s, as would be expected. These variations in recovery time may be explained by the different functional groups and chemical compositions that the proteins possess, as well as the different folded geometry of each of the proteins. Therefore, the molecular weight of the proteins alone may not be the sole contributor to its three-dimensional size. The protein's tertiary structure, or quaternary structure if it comprises subunits, may also influence the protein's mobility within a closed network.

The molecular composition of the capsule may also influence how the protein behaves and its potential mobility. The proteins we used are not inert molecules. Transferrin, albumin, and ceruloplasmin each contain functional groups that may interact with the capsule. Moreover, any chemical changes to the capsule, through oxidation or disease, may further alter the protein's behavior. In addition, we quantified only the diffusion of the heavy metal carriers into and within the anterior lens capsule. It is possible that the proteins would behave differently if the corresponding heavy metals were connected to the proteins. In the case of transferrin, it is possible that the protein was actually transporting iron, due to residual blood on the sample from dissection.

When developing the protocol for this study, we found that the FRAP signal intensity became variable when the samples had a postmortem time greater than 9 days. Because of this, only samples with a postmortem time of 9 days or less were included in this study. We analyzed the partition coefficient, half maximum recovery time, and diffusion coefficient as a function of postmortem time to determine if there was an effect. There was no relationship between the partition coefficient and the postmortem time for albumin, ceruloplasmin, or transferrin (p=0.556, p=0.779, and p=0.979, respectively). There was also no relationship between the half maximum recovery time or the diffusion coefficient and the postmortem time for albumin or ceruloplasmin (p=0.566 and p=0.441 for the albumin half maximum recovery time and diffusion coefficient, respectively; p=0.164 and p=0.176 for the ceruloplasmin half maximum recovery time and diffusion coefficient, respectively). With transferrin, however, the half maximum recovery time statistically significantly decreased (p=0.0108), and the diffusion coefficient statistically significantly increased (p=0.010) with the postmortem time. However, even after adjusting for these differences due to the postmortem time, there was still no relationship between the half maximum recovery time and age or the diffusion coefficient and age. Therefore, we can assume that the conclusions drawn in this study are independent of the postmortem time.

We did not control for lens epithelial cell viability during the storage period of the lenses. However, because the results are independent of the postmortem time, lens epithelial cell viability is not a source of error when calculating the properties of the lens capsule. Future experiments that examine protein diffusion within the lens fiber cells, however, would need to control for lens epithelial cell viability.

In summary, the results of this study indicate that the human anterior lens capsule becomes more permeable to heavy metal carriers with age. Age-related changes to the lens capsule may have important implications in the maintenance of overall lens biology.

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