Ion-Dependent Hydration and Transparency of the Corneal Stroma, a Polyelectrolyte Biogel

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Summary: The cornea is the clear outer window of the eye, composed primarily of dense regular connective tissue called the stroma. The tissue is composed of 70% water and parallel collagen fibrils surrounded by proteoglycans containing high concentrations of acidic and neutral glycosaminoglycans (GAGs) and contains ~2 – 10% cells by volume. When the surface membranes covering the stroma are damaged by injury or disease, the stroma swells due to the hydrophilic nature of the polysaccharides. At the same time, swelling spreads the collagen fibrils apart and the tissue loses transparency. We investigated the role of different salts in modifying swelling behavior and transparency loss. In saline (148 mM NaCl) solution, hydration increases as a multi exponential function of time: Initially the stroma gains hydration at a fast rate, then slows down. In the presence of divalent cations, swelling behavior significantly decreased, and the effect was weakly anion-dependent. Under imposed stromal swelling pressures, Ca^{++} and Mg^{++} inhibited hydration equally, and their effects were additive, indicating that they work by similar mechanisms. They were also concentration-dependent, with no apparent ceiling to the effect within normal biological ionic strengths. As stated above, Mg^{++} and Ca^{++} both dehydrate stromas and decrease swelling rates compared to saline, and as a stroma hydrates, it loses transparency: visible spectrophotometry of stromas bathed in NaCl lose transparency faster than those bathed in $MgSO_4$, because $MgSO_4$ solutions hydrate stromas slower. Stromas bathed in Ca^{++} hydrated slower than those bathed in NaCl, but interestingly, high (49 – 148 mM) concentrations of Ca^{++} caused transparency loss almost instantly. In hyaluronin (a collagen fibril-free polyanionic biogel), when similar concentrations of Ca^{++} were added, transparency also decreased. Our conclusion is that the dehydrating effects of Mg^{++} and low concentrations of Ca^{++} are due to shielding of electrostatic repulsion between anionic GAGs. The higher concentrations of Ca^{++} dehydrate by a combination of covalent binding to (precipitating) GAGs and/or shielding their electrostatic repulsions, and scatter light by precipitating GAGs and disrupting collagen fibril order. These techniques and ionic probes are useful for studying the structure and function of polyionic hydrogels and may be useful for modifying the materials properties of biological and synthetic gels.

Keywords: biopolymers; hydrogels; light scattering; swelling; UV-vis spectroscopy

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Introduction

The corneal stroma imbibes fluid, swells and loses transparency when denuded of its epithelium and endothelium. Hedbys[1] demonstrated the importance of polysaccharide hydration properties in stromal swelling. The sulfate and carboxylate groups in stromal polysaccharides are largely responsible for stromal hydration and may be responsible for cation binding. Green and Friedman[2] demonstrated that the stroma binds cations with high affinity and proposed that cation binding might at least partially inhibit stromal swelling physiologically, assisted by the ion transport systems of the epithelium and/or endothelium. Wiley and Fatt[3] provided limited evidence that some salts, including calcium chloride and magnesium chloride can thin stromas at normal swelling pressures. Ion-induced modulation of swelling pressure might result from alterations of the chain-chain repulsion between stromal glycosaminoglycans, but these have not been well documented. However, in model gels of polyelectrolytes, it is well known that ionic strength and valence affect the conformation, solubility and light scattering properties.[4] The aim of this study was to better characterize the effects of calcium and magnesium salts as experimental tools to moderate corneal stromal swelling rates and swelling pressure:hydration relationships. The effect of these cations, separately, together, and as a function of concentration and their counter-ion was examined.

Materials and Methods

Tissue Dissection:

Bovine eyes were obtained from a beef packing company on the day of dissection, transported at 0 - 4^0 C, and the corneal epithelia were scraped off with a #22 scalpel blade. The deepithelialized cornea and a 3 – 5 mm scleral skirt were removed with scissors and the lens, iris and ciliary body were removed with forceps. Vitreous body was collected for studies of hyaluronin, a negatively charged glycosaminoglycan (see below). Then the corneal endothelium was rubbed away with a paper towel and stromal buttons were punched out with a 19 mm diameter circular leather punch on a Teflon® block. During dissection, eyes and dissected corneas were both kept at 0 - 4^0 C, and corneal stromas were kept in a refrigerated moist chamber until experiments began 12 - 15 hours later.

Solutions Tested:

Phosphate buffered solutions of 148 mM sodium chloride, magnesium sulfate, calcium chloride, and mixtures thereof are given in Table 1.

Table 1. Solutions Tested.

Solution	mM NaCl	mM $MgSO_4$	mM $CaCl_2$
NaCl	148	-	-
4.9 mM $MgSO_4$	143.1	4.9	-
14.8 mM $MgSO_4$	133.2	14.8	-
49 mM $MgSO_4$	99	49	-
148 mM $MgSO_4$	0	148	0
4.9 mM $CaCl_2$	143.1	-	4.9
14.8 mM $CaCl_2$	133.2	-	14.8
49 mM $CaCl_2$	99	-	49
148 mM $CaCl_2$	0	-	148
24.6 mM Ca + 24.6 mM Mg	98.8	24.6	24.6

All solutions were buffered with 2 mM phosphate and adjusted to pH 7.3 – 7.5 on the day of use.

Vitreous body was centrifuged 2 hours at 10,000 x g in a Sorvall RC 5 C Plus centrifuge to separate the hyaluronin from the palletized collagen, cells and debris. The clear viscous negatively charged hyaluronin GAG was stored frozen in in borosilicate glass scintillation vials until use in spectrophotometry experiments designed to determine whether calcium or magnesium salts precipitate it. In those experiments, the hyaluronin was diluted 1:1 with either 296 or 98 mM $MgSO_4$ or $CaCl_2$ or 148 mM NaCl and spectra were run at 15 to 30 minute intervals. At the end of the experiments, the solutions were also inspected visually to observe if precipitates were present.

Stromal Swelling (Hydration) Rates:

Individual stromal buttons were placed in a borosilicate glass scintillation vial (in triplicate to n = 6) containing each bathing solution at 22 – 24^0 C and swirled gently to remove air bubbles on their surfaces. Stromas were removed every 30 minutes, blotted on a paper towel, weighed to 0.001 g on an analytical balance, and quickly returned to their bathing solutions. At the end of the third hour, the stromas were blotted, weighed, and placed in an 80^0 C desiccator oven for 24 - 48 hr until constant mass was attained. Stromal hydration (H) was calculated as H = (wet mass-dry mass)/(dry mass). A plot of H vs time was made based on

the wet weights and dry weights recorded for each tissue in each solution. Statistical comparisons were based on a two-tailed t test at 1 and 3 hours, or in the case of multiple comparisons, by analysis of variance and a Bonferoni's t test.

In several experiments, visible spectrophotometry was performed on stromas allowed to swell in the solutions in cuvettes. Those stromas were not weighed at each time point, because changing their position in the cuvettes changed the tissues' position in the light path, altering their spectra vs time.

Stromal Swelling Pressure vs Hydration:

Stromal buttons (1.9 cm in diameter) were pre-swollen for 30 min in a 50 ml beaker containing one experimental solution, then compressed with a series of standardized weights, in the same solution, in triplicate, between flat, 50 mm diameter sintered glass filter disks (Andrews Glass Co., Vineland, NJ) at swelling pressures of 33 to 150 mm Hg. The filter disks were held in polystyrene Petri dishes that contained 13 ml of the experimental solution. Glass spacers (Whitman Glass Shop, Carrollton, GA 30117) were placed over the upper glass filter so that weights could be placed on top of each Petri dish cover to compress the tissues. The weights were centered on each filter disk holder to impose the desired swelling pressures, bracketing the physiological range. As described previously[1,4,5], due to its lamellar structure, the stroma swells anisotropically, in its thickness direction, but not in diameter. At equilibrium hydration (obtained within 3 hours), stromas were blotted and equilibrium wet weights were measured on an analytical balance to the nearest 0.001g. Then the stromas were placed in an 80° C desiccator oven for 24 - 48 hr until constant mass was attained. Stromal hydration (H) was calculated as H = (wet mass-dry mass)/(dry mass). Log(swelling pressure) vs H was plotted for stromas bathed in each solution and slopes and (extrapolated) y intercepts were compared using analysis of covariance to determine whether or not a solution affected the hydration vs swelling pressure relationship.

Results

Swelling Rates With Divalent Cations:

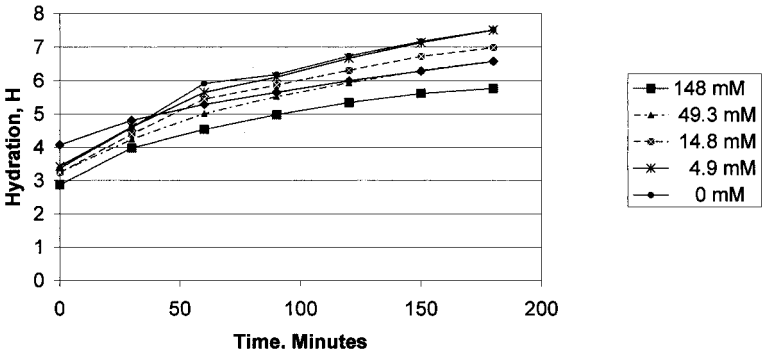

Figure 1. Swelling of bovine stromas in phosphate buffered saline (0 mM Mg^{++}) and 4.9 – 148 mM $MgSO_4$ in buffered saline. N = 3 – 6 corneas per point. Standard deviations for each point are approximately 0.3 to 0.5 hydration units.

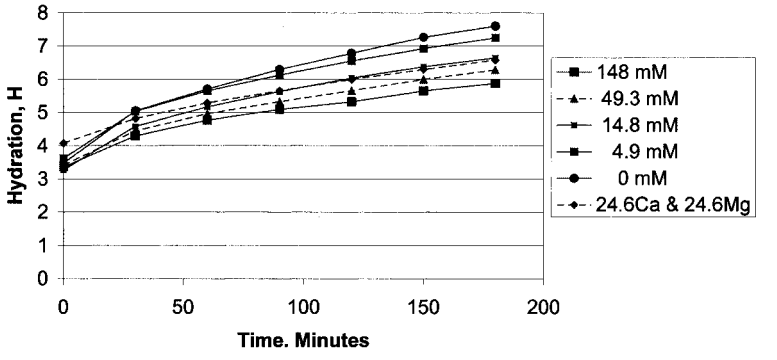

Figure 2. Swelling in phosphate buffered saline (0 mM Ca^{++}), 4.9 – 148 mM $CaCl_2$ and 24.6 mM $CaCl_2$ plus 24.6 mM $MgSO_4$ in buffered saline (additivity experiment). N = 3 – 6 corneas per point. Standard deviations for each point are approximately 0.3 to 0.5 hydration units.

Stromal swelling rates were rapid initially, almost doubling in the first hour, and decreased thereafter as previously reported (Doughty, 2003). Stromas swelled fastest in 148 mM NaCl, and slower in 49 – 148 mM $MgSO_4$ (Figure 1) and $CaCl_2$ (Figure 2) solutions. The effects of $MgSO_4$ and $CaCl_2$ were concentration-dependent and additive (Figure 2) rather than competitive ($p < 0.05$). Calcium and magnesium were not significantly different from each other on a molar basis at reducing stromal swelling rates.

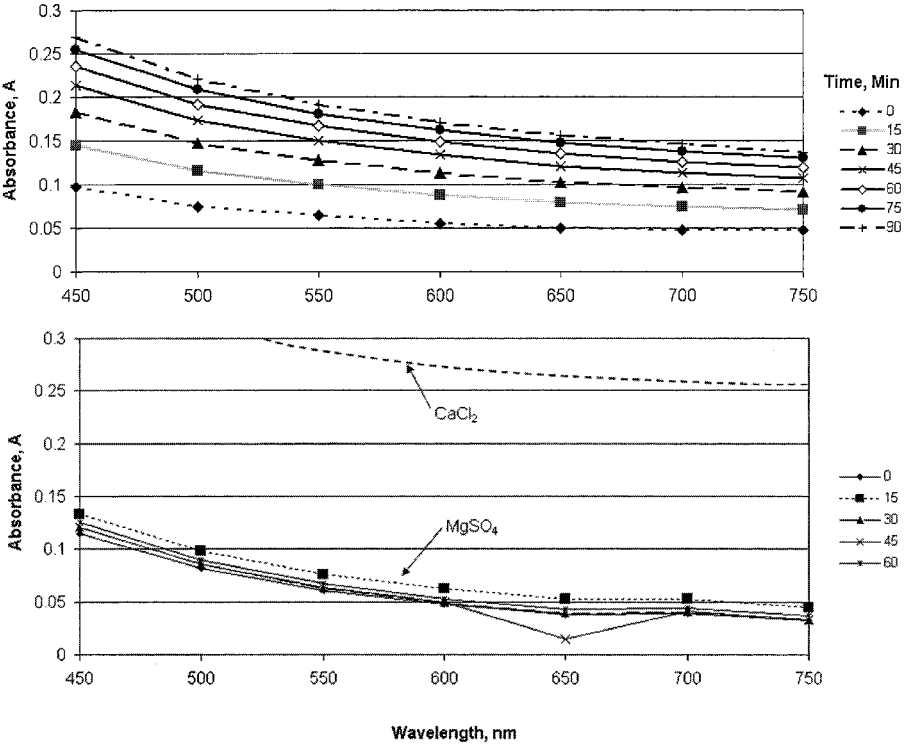

Figure 3. Time course of transparency loss during bovine stromal swelling in phosphate buffered saline (Upper graph) and 49 – 148 mM $MgSO_4$ and $CaCl_2$ in buffered saline (lower graph). Stromas bathed in 49 – 148 mM $CaCl_2$ (dashed line) lost transparency within seconds and transparency loss did not progress over 60 – 90 minutes.

In the stromal swelling experiments it was noted that stromas bathed in 49 – 148 mM $CaCl_2$ were translucent, whitish and rubbery, while those bathed in $MgSO_4$ were remarkably more transparent than those bathed in saline. This was confirmed with visible spectrophotometry in stromas bathed with NaCl, $MgSO_4$, and $CaCl_2$ (Figure 3). Stromas bathed in saline lost transparency over time. Those bathed in $MgSO_4$ lost transparency more slowly, and those

bathed in $CaCl_2$ lost transparency within seconds. At concentrations lower than 49 mM, calcium did not interfere with transparency, and spectra were not significantly different from saline spectra (data not shown).

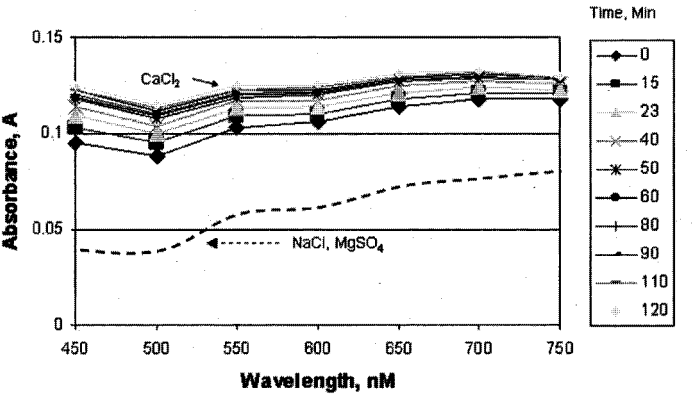

Figure 4. Time course of hyaluronin transparency after addition of 148 mM NaCl, $MgSO_4$ (dashed line; no change over time) or $CaCl_2$.

To determine the potential cause for the loss in light transmission (precipitation of GAGs vs precipitation of protein), spectra of hyaluronin mixed with NaCl, $MgSO_4$, and $CaCl_2$ were conducted. Spectra of hyaluronin mixed with NaCl, 4.9 – 14.8 mM $CaCl_2$, or 4.9 – 148 mM $MgSO_4$ were super imposable (Figure 4, dashed line), while those for hyaluronin with 49 – 148 mM $CaCl_2$ were much less transparent. After 120 minutes, the transparency improved over time, as a visible precipitate settled in the cuvette (data not shown). The precipitate was not identified chemically, but is in all likelihood calcium hyaluronate.

Stromal Swelling Pressure vs Hydration With Divalent Cations:

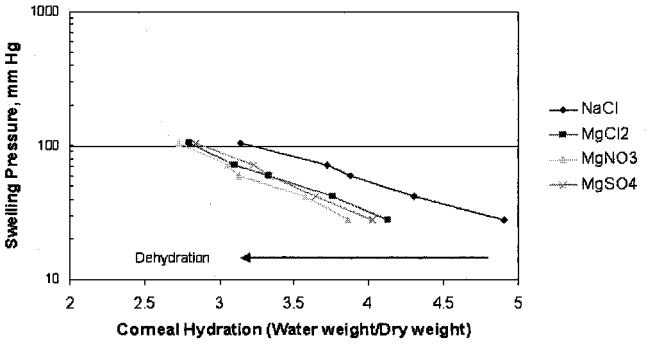

Figure 5. Swelling pressure vs hydration relationship for bovine stromas in buffered saline (148 mM NaCl) vs magnesium salts. N = 3 – 6 corneas per point. Standard deviation per point is 0.3 to 0.5 hydration units.

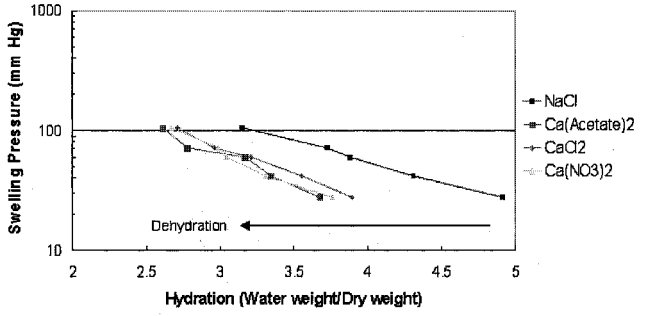

Figure 6. Swelling pressure vs hydration relationship for bovine stromas in buffered saline (148 mM NaCl) vs calcium salts. N = 3 – 6 corneas per point. Standard deviation per point is 0.3 to 0.5 hydration units.

In saline and low concentrations of $MgSO_4$ and $CaCl_2$, the relationship of hydration as a function of log(swelling pressure) was a straight line of similar slope and (extrapolated) y intercept. In 148 mM $MgSO_4$ (Figure 5) and $CaCl_2$ (Figure 6), the stromas dehydrated and the slope steepened significantly ($p < 0.05$) compared to those in saline, such that the dehydration effect was greater at low swelling pressures, and less at higher ones. $MgSO_4$ was a slightly, but not a significantly poorer dehydrator than $CaCl_2$. Other magnesium and calcium salts dehydrated stromas similarly (Figures 5 and 6), regardless of the anion present, and in the case of magnesium, regardless of the anion's valence and molarity.

Discussion

Effects of divalent cations on stromal hydration have previously been reported,[3,5] but their details have not been studied. In our study, we found that calcium and magnesium ions decrease stromal swelling in a concentration-dependent manner, and both are additive (not competitive) on a molar basis, independent of the anion concentration (e.g., chloride vs. sulfate). For magnesium ions, based on what is known about stromal swelling properties, this is probably due to electrostatic shielding of GAG repulsion by the bulky divalent cations. For calcium ions, the effect may in part be due to electrostatic shielding and in part due to covalent binding to the negatively charged GAGs at higher (49 – 148 mM) concentrations.

Both magnesium and calcium ions kept stromas thinner and less hydrated than saline. However, magnesium ions kept stromas more transparent, and calcium kept them more translucent than saline. The findings with calcium are a counter-example to what has been historically accepted in the ophthalmic literature (i.e., dehydrated corneas are usually more transparent than overly hydrated corneas). Based on our spectrophotometric data and observation of $CaCl_2$ and hyaluronin, a polyanionic GAG, high concentrations of calcium salts (49 – 148 mM) precipitate with GAGs. Hyaluronin's spectra was unaffected by $MgSO_4$. It is unclear whether calcium also precipitates with collagen or other stromal proteins. However, with high Ca^{++}, the stromal spectra indicate a disordered stromal collagen fibril structure: in contrast with the hyaluronin spectra, the stromal spectra do have a much larger absorbance at shorter wavelengths, consistent with long range, wavelength-dependent collagen fibril light scattering.[4] Stromal spectra in $MgSO_4$ demonstrated less light scatter (and better collagen fibril order) than in saline.

Stromal hydration rates are a measure of *kinetic energy* of the gel's interaction with water and ions during swelling. In contrast, stromal swelling pressure is a measure of the *potential energy* of the system that causes stromal hydration. The stromal swelling pressure experiments demonstrate that divalent cations can modify the swelling pressure to hydration relationship, complementing our findings that divalent cations inhibit stromal swelling rates. Swelling pressures imposed on the stroma are done to achieve equilibrium hydrations. Under imposed swelling pressures, calcium and magnesium were significant dehydrators compared to saline ($p < 0.05$), and the dehydrating effects were anion-independent. Thus, divalent cations decrease both the kinetic and potential energy in this biogel.

Being a dense regular connective tissue, the cornea swells when its epithelium and endothelium are removed, damaged, and when refrigerated during eye bank storage prior to transplantation. It is possible that these or other divalent (or even polyvalent) ions could be used to limit swelling of corneas under eye bank conditions if they could be made to penetrate the corneal epithelium and endothelium. This could be achieved if a corneal "button" was cut or punched out of the eye and stored with its bare stromal edge exposed to permeant polyvalent cationic salts in the solution. Considering the high calcium and magnesium concentrations needed to produce significant efficacy (49 mM Ca^{++} or Mg^{++}, or 24.6 mM Ca^{++} plus 24.6 mM Mg^{++}), alternative polycations need to be examined. Otherwise, toxicology screening of Ca^{++} and Mg^{++} is warranted. Divalent cations may also be useful for controlling swelling in other tissues, such as tendon or ligament. They could also prove to be useful research tools for reversibly (or irreversibly) altering anionic GAG repulsion without disrupting (or with disrupting) collagen structure or other features of the tissue.

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