DOI: https://doi.org/10.1016/j.jcrs.2015.09.032

Rate of riboflavin diffusion from intra-stromal channels prior to corneal cross-linking (CXL)

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No author has a financial or proprietary interest in any material or method mentioned.

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Keywords: Diffusion, intra-stromal channels, riboflavin, cross-linking, cornea, keratoconus

Abstract

Treatment time for Corneal cross-linking (CXL) can potentially be reduced by use of riboflavin diffusion from intra-stromal channels rather than axial diffusion with epithelium on or off. Here, the purpose is to determine the rate of transverse diffusion for six riboflavin solutions via intra-stromal corneal channels. Riboflavin diffusion was realized through the manual creation of an intra-stromal channel in whole-mounted porcine eyes. Fluorescence light distributions were imaged under UV illumination. The rate of diffusion for each concentration was monitored for a total of 30 min using an optical setup with a CCD camera with a bandpass filter (central wavelength 550nm and 40nm bandpass). An isotropic corneal stroma was modelled numerically with the aim of analysing the effectiveness of diffusion of riboflavin from an intra-stromal channel as a function of time for different diffusion constants and boundary conditions. The numerical results were compared to the experiments and to an expected typical axial diffusion coefficient for riboflavin of $D_0 = 6.5 \times 10^{-5}\,\mathrm{mm}^2/\mathrm{sec}$ thereby allowing determination of the effective diffusion coefficient for each case. Experimental results were in all cases found to be higher than for axial diffusion. Two isotonic solutions containing 0.1% riboflavin display a similar diffusion rate, correlating to the numerical model with a diffusion constant of $5D_0 = 32.5 \times 10^{-5}\,\mathrm{mm}^2/\mathrm{sec}$. Furthermore, the results show that hypotonic solutions and TE have a higher diffusion coefficient approaching $10D_0 = 65.0 \times 10^{-5}\,\mathrm{mm}^2/\mathrm{sec}$, demonstrating an order-of-magnitude increase compared to the typical diffusion coefficient found in standard CXL with riboflavin drops applied repeatedly to the cornea. This study shows that riboflavin has a faster stromal diffusion when injected into a corneal channel than when applied as drops to the anterior surface.

Numerical modelling of the diffusion process allows optimization of the channel structure for any specific choice of riboflavin. Further research is required to model the role of oxygen diffusion in the proposed channel configuration.

Introduction

Corneal cross-linking (CXL) is an established method for the treatment of keratoconus[1]. The standard CXL protocol consists of riboflavin (vitamin B2) applied to a de-epithelized cornea for 30 min. to facilitate diffusion into the stroma, followed by exposure to UV-A (365nm) at 3mW/cm² for an additional 30 min. Riboflavin plays a key role in the CXL process as it triggers the formation of molecular crosslinks that halt the progression of the condition and lead to stabilization of corneal curvature. Apart from its active role in CXL, riboflavin acts as a safety barrier that hinders UV exposure of the posterior parts of the eye[2]. Riboflavin is a hydrophilic molecule[3,4], allowing for the diffusion process to naturally occur through the application of drops onto the cornea after epithelial removal. Because of the tight junctions between epithelial cells, riboflavin cannot penetrate fully unless the epithelium is removed prior to application[5]. Effective diffusion to a stromal depth of ~300µm is 30 min., as indicated by the standard CXL "Dresden Protocol" [6]. A clinical limitation of the standard treatment is epithelium removal leading to pain for the patient and a potential higher clinical risk after the treatment during the phase of epithelium closure[7].

New methods of riboflavin delivery and introduction of new riboflavin solutions prior to CXL such as trans-epithelium[8]; Iontophoresis[9,10], femtosecond laser channels[11], and accelerated CXL[12] have been investigated. Studies have investigated the UV absorption coefficient of riboflavin and found a linear correlation of concentrations up to 0-0.5% and its effect on diffusion in the corneal stroma, finding a higher riboflavin concentration 400µm the stroma[13,14]. This suggests that changes in the times to administer at the corneal surface are linked to the riboflavin concentration. The concentration distribution depends on the reservoir of riboflavin available at the corneal surface (e.g layer thickness and dropping intervals) [15] .

Thus, standardization to the clinic dropping scheme related to the application of riboflavin is important to achieve consistency in the treatment outcome as well as in optimizing the treatment itself.

In this paper, we report on measurements of riboflavin diffusion using intra-stromal channels created by means of a mechanical stromal instrument (Suarez Spreader) in whole-mounted post-mortem porcine eyes. The use of fluorescent imaging along with numerical modelling allows determination of effective diffusion coefficients under different conditions. The use of corneal channels could potentially allow surgeons to apply the riboflavin into the stroma without epithelium removal.

Methods

Experimental Setup

The rate of diffusion was monitored using an optical setup with a CCD camera (Thorlabs Inc, DCC 1240C). A bandpass filter (central wavelength 550nm and bandpass 40nm) was attached to the camera to measure riboflavin fluorescence when exposed to UV (wavelength 365nm ± 10 nm) to image only the fluorescent light. The layout of the system is shown schematically in Figure 1.

Preparation of eyes

Enucleated porcine eyes were obtained 2 -3 hours post-mortem from the local abattoir. Eyes were stored at 4ºC and used within 6 hours. All corneas were found to be clear, with no

presence of corneal scarring or opacities. Balanced salt solution (BSS) was inserted through the optic nerve to enhance and maintain the natural shape, and the eye was placed in a holder with the cornea facing upwards. The epithelium was removed due to loosening post mortem using an epi-hook and a small incision was created in the cornea to allow for the Suarez Spreader (Mediphacos Ltd, Brazil).

The mechanical channel was attempted to have a diameter of 5.7 mm. The incision channel was created in the upper half of the stroma ideally at 200 microns. Riboflavin (0.2ml) was injected into the channel using a lacrimel cannula (Visitec®). The injection was performed until the channel was entirely filled by the riboflavin solution at which point no further riboflavin re-injection was applied during the imaging sequence in order to mimic a clinical situation. Each experiment was repeated multiple times with similar diffusion rates found.

Imaging Method

The cornea was illuminated with UV light at 365nm wavelength (UV-X prototype, IROC Innocross, Zurich Switzerland,) 6 mW/cm^2 in order to excite the fluorescence of the riboflavin in the channel. An image was taken immediately (approx. 2 min. after having injected riboflavin into the channel) and repeated every 10 min. for a total of 30 min. beginning at time 0 in order to measure a baseline of riboflavin in the corneal channel.

Riboflavin Formulations

Six riboflavin formulations (Medio-Haus-Medizinprodukte GmbH, Germany) were tested in this study:

A. Mediocross®-R Isotonic (riboflavin-5-phosphate 0.23% without dextran T-500).
B. Mediocross®-D Isotonic (riboflavin-5-phosphate 0.1% with 20% dextran T-500).
C. Mediocross®-M Isotonic (riboflavin-5-phosphate 0.1% with 1.1% Hydroxypropyl Methycellulose, HPMC).
D. Mediocross®-H Hypotonic (riboflavin-5-phosphate 0.1% without dextran T-500)
E. Streuli Hypotonic (riboflavin-5-phosphate 0.5% without dextran T-500)
F. Mediocross®-TE Trans-epi (riboflavin-5-phosphate 0.25% with 1.2% Hydroxypropyl Methycellulose (HPMC) & 0.01% Benzalkonium chloride (BAC).

Image processing

The diffusion of riboflavin from the channel can be determined experimentally. Figure 2 shows results comparing hypotonic and isotonic diffusion with two different concentrations. A difference in the diffusion rate can clearly be seen. Using Matlab™ software (Mathworks Inc,R2013b), 100 radial cuts were made in the recorded images covering a 180º arc (left side of images in Figure 2) whereby the Full Width at Half Maximum (FWHM) of the riboflavin distribution for each instant was determined. This averaging of the results allows for more reliability of diffusion determinations.

OCT Analysis

3-Dimensional Ocular Coherence Tomography (OCT-2000, Topcon, UK) was performed on one eye to estimate the approximate channel depth in the stroma, and to confirm that channels could be successfully made by the procedure. The average porcine corneal thickness is 800-1200µm. Figure 3 shows OCT images of the created channel. Image analysis shows the channel closed pre- riboflavin insertion (80-81µm), before increasing in diameter from the force of riboflavin entering the channel (146-148µm).

Diffusion Model

The diffusion is calculated using Fick's 2nd law in 3-D:

$$\frac{\partial c(x,y,z;t)}{\partial t} = D\nabla^2 c(x,y,z;t)$$

where $c(x,y,z;t)$ is the riboflavin concentration at any point across the corneal model at time *t*, and D is the characteristic diffusion constant which, in the case of axial-corneal diffusion for common epi-off CXL has been found to equal $D_0 = 6.5\times10^{-5}\,\mathrm{mm}^2/\mathrm{sec}$ in previous studies[13,15,16].

An isotropic corneal stroma model was made to solve Ficks 2nd law of diffusion using COMSOL™ (COMSOL Inc) with the aim of analysing the effectiveness of diffusion of riboflavin from an intra-stromal channel as a function of time for different diffusion constants and boundary conditions, seen in Fig. 4 and 5. The rotationally-symmetric corneal model has

a diameter of 12 mm and a thickness of 1.0 mm and the surgical incision (applied halfway into the stroma) has a 5.7 mm diameter with a rectangular 0.6 mm × 0.2 mm cross section. Analysis was implemented with a finite amount of riboflavin applied to the channel as initial condition (with no riboflavin present elsewhere at Time t=0) and then diffused over time into the stroma. This is the case used in the experiments and would be preferred in a clinical setting. Figure 4 shows the simulated case of a finite supply of riboflavin application to the channel wherefrom it spreads over. Note that a sector has been removed for visualization of the diffusion inside of the rotationally-symmetric stroma and channel.

The numerical analysis used the standard diffusion coefficient (or multiples thereof) of riboflavin seen in previous work[15], and calculated the best fit to the experimental results.

Experimental Results

Experimental analysis in Figure 6 show an increase in FWHM of the riboflavin between T=0 min. and T=30 min for all formulations. Over the 30 min interval, isotonic 0.23% (Fig.6a), Dextran (Fig.6b), and HPMC (Fig.6c), the estimated FWHM increased by 31%, 25% and 31% respectively. For hypotonic 0.1% (Fig.6d), 0.5% (Fig.6e) and TE riboflavin (Fig.5f), the width increased by 44%, 45% and 42% respectively for T=0 to T=30min.

Both isotonic solutions containing 0.1% riboflavin shown in Figures 6b and 6c have a similar diffusion rate seen to spread out of the channel as the normalized intensity at R=0 shows 0.2 (Dextran), and 0.3 (HPMC) at T=30 min., correlating to the theoretical model of $5D_0 = 32.5 \times 10^{-5}\ \mathrm{mm}^2/\sec$. A higher rate of diffusion over 30 min. can be seen in isotonic 0.23% (Figure 5a) which peaks at 0.4 for R=0. The channel acts as a chamber for riboflavin absorption which is highlighted more in hypotonic when compared to isotonic solution.

Results show that hypotonic solutions and TE (Figure 6d, 6e, and 6f) have a higher diffusion coefficient of close to $10D_0 = 65.0 \times 10^{-5}\,\mathrm{mm}^2/\sec$ which is an order of magnitude higher than in standard axial diffusion into the stroma via corneal drops. At T=30min. both hypotonic solutions (0.1%, 0.5%) show a wider spread than for isotonic 0.1%.

Discussion

The objective of the study was to investigate diffusion in the corneal stroma through mechanically created stromal channels for riboflavin delivery. Six solutions of riboflavin with varying concentrations were measured under fluorescence to observe diffusion over time set by the standard CXL protocol[6], in order to enhance treatment performance or outcomes without the need for epithelial removal.

Two isotonic solutions containing 0.1% riboflavin display a similar diffusion rate, correlating with the numerical model having a diffusion constant of $5D_0 = 32.5 \times 10^{-5}\,\mathrm{mm}^2/\sec$, i.e five times higher than previously assumed axial diffusion rate[13,15,16]. Furthermore, the results show that hypotonic solutions and TE have a higher diffusion coefficient approaching $10D_0 = 65.0 \times 10^{-5}\,\mathrm{mm}^2/\sec$, demonstrating an order of magnitude increase compared to the diffusion coefficient found for standard CXL with riboflavin drops applied repeatedly to the cornea.

Numerical and experimental results show that each riboflavin concentration has a higher diffusion rate when applied to the channel than if applied to the front surface of the corneal stroma. Also, a difference in diffusion structure based on each riboflavin formulation can be seen. Other boundary conditions have also been examined in COMSOL™ such as a

continuous supply of riboflavin over time to the channel. This has no significant impact on the predicted results (except that the highest concentration coincides at all times with the location of the channel) and highly similar diffusion coefficients were found.

Experimental results show a variation in structure based on each riboflavin formulation tested. 0.01% Benzalkonium Chloride 0.01% (BAC) is commonly used for trans-epithelial (TE) CXL[17,18]. Its function is to loosen the tight junctions between epithelial cells without the need for removal in order for the hydrophilic riboflavin molecule to pass into the corneal stroma before exposure to UV, reducing pain and discomfort for the patient[19]. Using TE riboflavin via a corneal channel could increase diffusion into the stroma as seen in Figure 6f by the 42% increase in FWHM between T=0 min and T=30 min.

OCT images demonstrate a 3D analysis of the channels behaviour when filled with riboflavin solution. The diameter of the channel is increased after insertion of riboflavin, followed by a decrease in channel width over 30 minute as the riboflavin diffuses into the stroma.

Previous studies found a diffusion coefficient $D_0 = 6.5 \times 10^{-5}\,\mathrm{mm}^2/\mathrm{sec}$ for 0.1% riboflavin with 20% dextran[6]. Our findings show that the rate of diffusion from an intrastromal channel is approximately 5× higher. Experimental results indicate a 25% FWHM increase over 30 min. This is likely due to dextran being of high viscosity, preventing the riboflavin from spreading out of the channel at a fast rate. Schmidinger et al[20] found riboflavin phosphate solution (HPMC) to have a lower absorption coefficient, a thinner meniscus and a viscosity dissimilar to isoosmolar riboflavin with 20% dextran. A difference in diffusion rates between dextran and HPMC is clearly seen as the FWHM increases by 42% for HPMC between 0 and 30 min.

Hypoosmolar riboflavin was introduced to the CXL procedure in order to swell the cornea before exposure to UVA for eyes with thin corneas (Hafezi et al, 2009) for safety of posterior structures of the eye. As the hypotonic is injected into the channel, this may swell the stroma and therefore increase the space between lamellae enhancing the transverse diffusion process. Also, hypoosmolar high concentration of 0.5% increases the availability of riboflavin molecules. When comparing the FWHM increase seen in Figure 6, TE & hypotonic results correspond to this stromal swelling, allowing for a reasonably large spread of riboflavin over 30 min. Figure 6e shows a diverse distribution of diffusion compared to other formulations, perhaps due to the high concentration of riboflavin.

Typically, TE CXL would be preferable in a clinical setting. Compared to this, the experimental limitations in this study included the removal of the epithelium in porcine eyes due to alterations post mortem. However, keeping the epithelium intact in a clinical setting would be desirable. Also, an estimation of corneal depth was recorded through OCT as an example of the diffusion experienced experimentally (Figure 3, 179-349µm).

During standard CXL, drops of riboflavin are applied to the corneal surface after epithelial removal. This agrees with the assumption that riboflavin will diffuse into the corneal layers in a homogenous distribution, covering an 8mm treatment area. When using corneal channels, we visibly observed an axial and lateral diffusion process, which increased over time.

The channels created in this study have demonstrated that riboflavin can be efficiently delivered into the stroma as a method of bypassing the epithelium. However, we do not know the biomechanical effect of CXL using stromal channels in terms of efficacy. During CXL, riboflavin and UV bond together in order for the creation of cross-links, resulting in biomechanical strengthening[1]. The role of oxygen (O_2) must also be taken into account during the CXL process. By applying drops to the surface of the cornea using the Dresden protocol,

atmospheric O_2 is taken up by the UV light and reacts with the riboflavin[22]. When cross-linking through a stromal channel, the O_2 concentration available would be lower than on the surface of the cornea and therefore may reduce the CXL effect. Future biomechanical measurements and the use of Finite Element Modelling (FEM) of the CXL effect should examine this in more detail.

Epithelial-on[18,19] or trans-stromal disruptors[9,10] without the need for epithelial removal would be preferable for both patient and surgeon in a clinical setting. However, reducing application time may be patient dependent with isotonic displaying a lower diffusion rate then hypotonic. In-vivo studies for the above methods are desirable. Future investigative studies using fluorophotometry[16] or optoacoustic methods[23] in order to learn more about the diffusion process whilst monitoring corneal depth in the lateral and axial dimensions of the stroma would be preferable.

Conclusion

The installation of riboflavin through mechanically created channels has been shown. The diffusion rate shows dependency on riboflavin formulations when compared to literature of standard diffusion dropping intervals. This study showed riboflavin has a faster lateral and axial diffusion when injected into a corneal channel. Numerical modelling of the diffusion process based on experimental results also allows optimization of the channel structure, indicating riboflavin diffusion is concentration dependant. Future work on the role of oxygen and photon distribution in combination with biomechanical simulation is required**.** Finally, it should be stressed that even when the diffusion rate is higher from a channel than when riboflavin is applied via drops, it remains to be studied how efficient it will promote the formation of cross-links during CXL. The latter can only be answered through mechanical stress-strain analysis.

Acknowledgements

The authors are grateful for financial support from Irish Research Council Enterprise Partnership Scheme (EPSPG/2013/598) and Wellington Eye Clinic, Sandyford, Dublin 18, Ireland.

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Figures

Figure 1. Schematic diagram of the system: CCD: Charge-coupled colour camera, Bandpass filter, f1 and f2 achromatic lenses, M - Mirror, UV-A radiation source at 365 nm, Ultra-violet light wavelength.

Figure 2. Experimental results of diffusion over time under the influence of corneal channels in porcine eyes using two different concentrations of hypotonic and isotonic riboflavin.

Figure 3. Example of a channel in a porcine cornea prior to (A), and after riboflavin instillation (B).

Figure 4. Stromal model showing riboflavin diffusion as a function of time (horizontal) for three different diffusion rates (vertical) equal to D_0, $5D_0$ and $10D_0$. In the model a finite supply of riboflavin is assumed in the channel at T=0. All sub-images have been normalized individually to ease visualization at increasing time.

Figure 5. Radial averages of the calculated riboflavin diffusion at increasing time for diffusion where each plot has been normalized.

Figure 6. Radial averages of fluorescent images of the corneal stroma following injection of riboflavin in time increments of 10min. from 0 to 30min. using 6 different riboflavin solutions where each plot has been normalized.

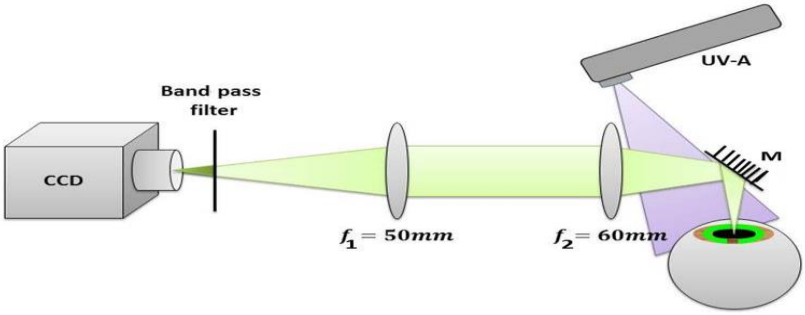
UV-A
Band pass filter
CCD
M
$f_1 = 50mm$
$f_2 = 60mm$

Fig. 1

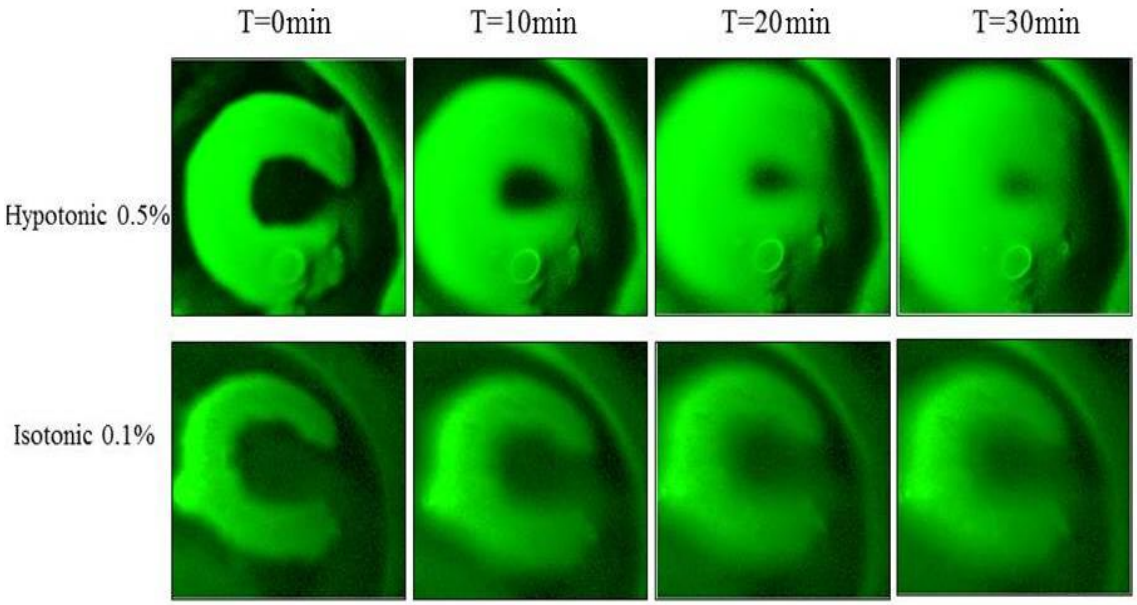
T=0min
T=10min
T=20min
T=30min
Hypotonic 0.5%
Isotonic 0.1%

Fig. 2

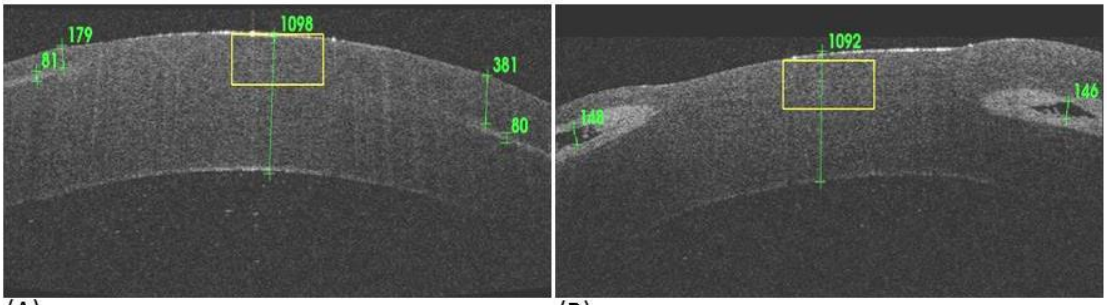
179
81
1098
381
80
1092
148
146
(A)
(B)

Fig. 3

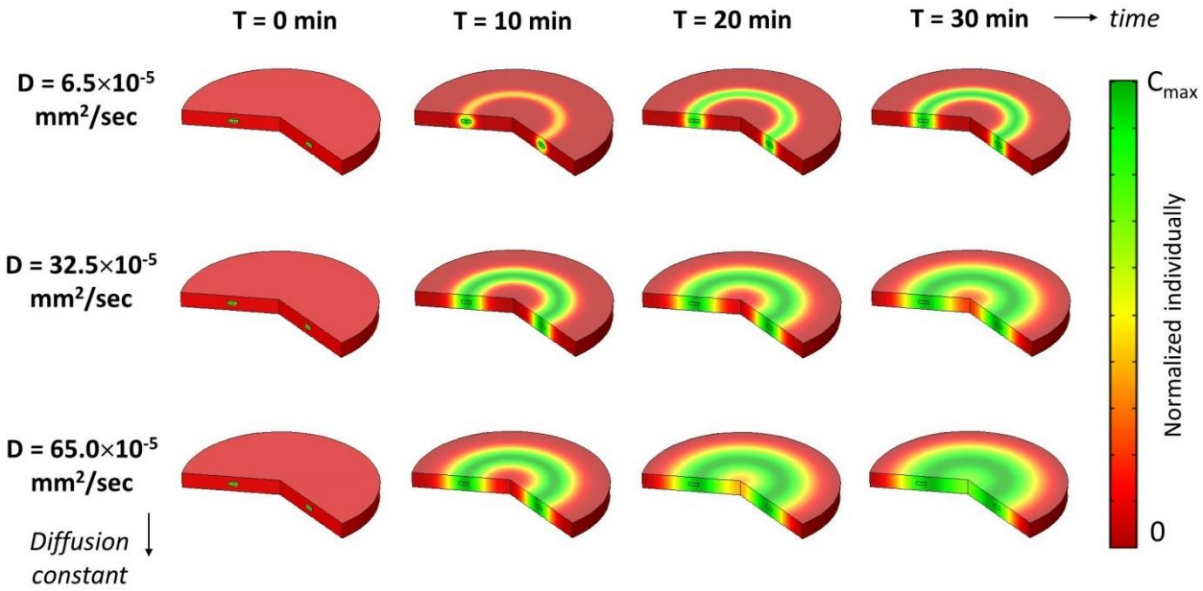
T = 0 min
T = 10 min
T = 20 min
T = 30 min
time
D = 6.5×10-5 mm²/sec
D = 32.5×10-5 mm²/sec
D = 65.0×10-5 mm²/sec
Diffusion constant
Cmax
Normalized individually
0

Fig. 4

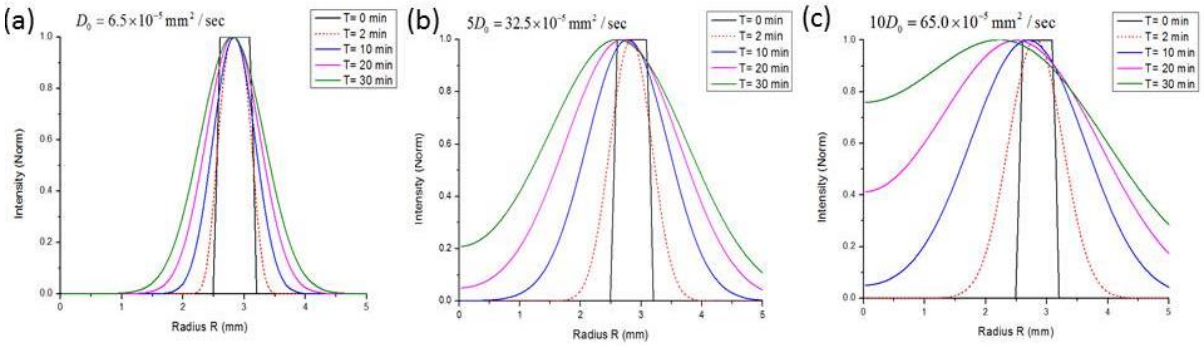
(a)
$D_0 = 6.5\times10^{-5}$ mm^2 / sec
(b)
$5D_0 = 32.5\times10^{-5}$ mm^2 / sec
(c)
$10D_0 = 65.0\times10^{-5}$ mm^2 / sec
T= 0 min
T= 2 min
T= 10 min
T= 20 min
T= 30 min
Intensity (Norm)
Radius R (mm)

Fig. 5

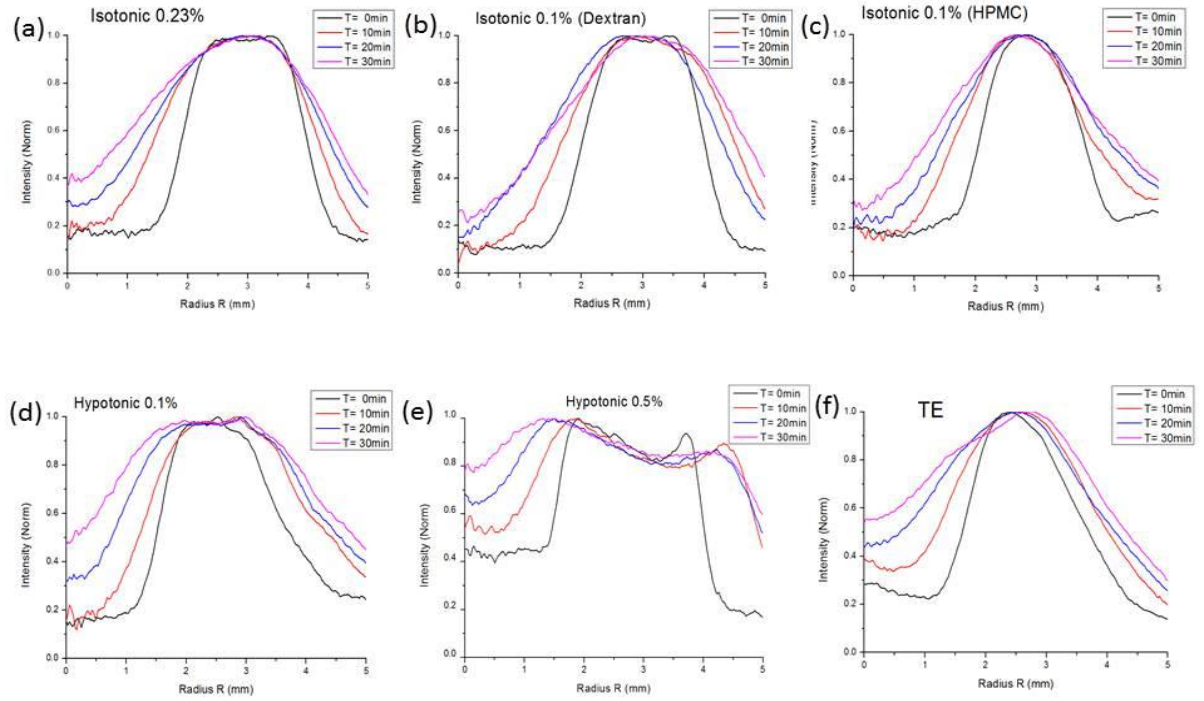
(a) Isotonic 0.23%
(b) Isotonic 0.1% (Dextran)
(c) Isotonic 0.1% (HPMC)
(d) Hypotonic 0.1%
(e) Hypotonic 0.5%
(f) TE
T= 0min
T= 10min
T= 20min
T= 30min
Intensity (Norm)
Radius R (mm)

Fig. 6