Published in final edited form as:

Ann Biomed Eng. 2012 December ; 40(12): 2551–2558. doi:10.1007/s10439-012-0606-4.

NUTRIENT TRANSPORT IN HUMAN ANNULUS FIBROSUS IS AFFECTED BY COMPRESSIVE STRAIN AND ANISOTROPY

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Abstract

The avascular intervertebral disc (IVD) receives nutrition via transport from surrounding vasculature; poor nutrition is believed to be a main cause of disc degeneration. In this study, we investigated the effects of mechanical deformation and anisotropy on the transport of two important nutrients – oxygen and glucose – in human annulus fibrosus (AF). The diffusivities of oxygen and glucose were measured under three levels of uniaxial confined compression – 0%, 10%, and 20% – and in three directions – axial, circumferential, and radial. The glucose partition coefficient was also measured at three compression levels. Results for glucose and oxygen diffusivity in AF ranged from 4.46×10^{-7} to 9.77×10^{-6} cm²/s and were comparable to previous studies; the glucose partition coefficient ranged from 0.71 to 0.82 and was also similar to previous results. Transport properties were found to decrease with increasing deformation, likely caused by fluid exudation during tissue compression and reduction in pore size. Furthermore, diffusivity in the radial direction was lower than in the axial or circumferential directions, indicating that nutrient transport in human AF is anisotropic. This behavior is likely a consequence of the layered structure and unique collagen architecture of AF tissue. These findings are important for better understanding nutritional supply in IVD and related disc degeneration.

Keywords

glucose; oxygen; nutrition; diffusion; partitioning; solubility; intervertebral disc

INTRODUCTION

The intervertebral disc (IVD) plays the important role of distributing loads in the spine, as well as allowing for spinal flexibility. The disc comprises the central, gel-like nucleus pulposus (NP) region surrounded by the layered, collagenous annulus fibrosus (AF). The highly organized extracellular matrix (ECM) of the disc plays an integral role in mechanical functioning. Within the ECM is a small population of cells, making up only about 1% of the disc by volume⁴⁴, and responsible for maintaining the health of the matrix. Because the IVD

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is avascular, nutrients must be provided by the surrounding blood vessels. Hence, cells rely on transport processes through the ECM to receive vital nutrients, including oxygen and glucose.

Transport properties of solutes in tissues include solute diffusivity and solubility (or partitioning). Diffusivity, or the diffusion coefficient, of a solute is a measure of the rate at which a solute moves through a tissue driven by a concentration gradient. Solute partitioning, or solubility, relates the concentration of solute inside the tissue relative to that in the surrounding fluid at equilibrium, and therefore governs the quantity of solute within a tissue. These solute transport properties are governed by characteristics of both the solute and the tissue ECM. This includes solute size and charge, as well as pore size of the matrix, which is directly related to tissue hydration and structure.^{8,29} It has generally been found that solute transport is inversely proportional to solute size, see review.¹⁹ Furthermore, transport properties of solutes have also been shown to be directly proportional to tissue water content; an increase in water content corresponds to an increase in the pore size of the tissue.^{1,2,5,10,16,20,22,36,46}

The IVD has a unique architecture and composition, containing primarily water, along with collagen and proteoglycans. The AF is composed of 15 to 25 concentric lamellae made up of collagen fiber bundles that run parallel to each other within each lamella, but opposite to those in adjacent lamella.^{14,26} Recent studies on animal and human AF tissue have revealed a unique hole or tube-like morphology with “microtubes” extending along the direction of collagen fiber bundles.^{18,20,41} Few studies have investigated the anisotropic nature of solute diffusivity in IVD tissues; see recent review in Travascio et al (2009).⁴¹ It has been suggested that this unique structure contributes to the direction-dependent diffusion in the disc. However, the anisotropic transport of nutrients in human IVD tissues has not previously been investigated.

As noted, tissue water content plays a primary role in governing transport properties in cartilaginous tissues; therefore, factors that alter water content also influence solute transport in the disc. For instance, mechanical loading, which occurs throughout daily activities in the disc, has been shown to vary the water content, chemical composition, and nutrient levels in the IVD.^{2,5,16} Tissue compression leads to fluid exudation, and a subsequent decrease in water content and reduction in pore size. Previous studies have shown that compression results in decreased rates of diffusion in the IVD, for both human and animal discs.^{3,10,20,46} Therefore, deformation of the disc during normal activity may limit nutritional supply because of decreased transport in the tissue; this can be detrimental to disc cells and the tissue health as a whole.

Each year, up to 45% of Americans experience low back pain, resulting in annual expenditures exceeding \$50 billion.³¹ Although the origins of low back pain remain unclear, symptoms have been strongly associated with degeneration of the IVD. Lack of proper nutritional supply to the disc has been implicated as a trigger in the onset of degeneration.^{5,16,17,30,42} Currently, there is a lack of adequate quantitative knowledge of transport properties of important nutrients in human IVD tissues, particularly under mechanical loading conditions. Here, we investigated the transport of oxygen and glucose in human IVD. We hypothesized that nutrient transport in human AF is anisotropic, owing to the unique collagen architecture in the tissue, and strain-dependent, because of the fluid exudation and reduction in pore size resulting from compression. In order to test these hypotheses, we measured the diffusion coefficients of glucose and oxygen in human AF tissue at three levels of compressive strain and in the three principal directions using a one-dimensional diffusion experiment. Additionally, we measured the strain-dependent partition coefficient (i.e., solubility) of glucose in human AF tissues.

MATERIALS AND METHODS

Specimen Preparation

A total of four human lumbar spines (ages 40, 41, 47, 63) were obtained from the University of Miami Tissue Bank. From each, the L2–L3 disc was harvested and graded morphologically using the Thompson grading scheme³⁹; two discs were grade I, one was grade II and one was grade III. For diffusivity tests, cylindrical AF specimens (diameter = 6 mm) were excised using a stainless steel corneal trephine (Biomedical Research Instruments, Inc., Silver Spring, MD) and cut to a height of ~0.5 mm with a sledge microtome (Model SM2400, Leica Instruments, Nussloch, Germany) with freezing stage (Model BFS-30, Physitemp Instruments, Inc., Clifton, NJ). Specimens were prepared in each of three principal directions: axial, circumferential, and radial (Fig. 1). For glucose, specimens were prepared from all four discs, for a total of 16 specimens in each direction (n=16), while specimens were only taken from three discs (two grade I discs and one grade III disc) for oxygen measurements (i.e., n=12 in each direction). Three tests, corresponding to three levels of compression (~0%, 10% and 20%), were performed on each sample.

For partition coefficient measurements, AF specimens were prepared using the same methods as those for diffusivity specimens (diameter = 8 mm; height ~ 2.0–2.5 mm) from the same L2–L3 discs. Nine specimens were excised from each disc, for a total of thirty six (n=36) specimens, and divided into three strain groups: 0%, 10% and 20%; one test was performed on each specimen. Only the glucose partition coefficient was measured in this study; that of oxygen was assumed to be unity given the small size of the oxygen molecule.

Diffusivity Measurements

A one-dimensional diffusion experiment, similar to previously described methods,^{20,28,29,46} was used to measure diffusivity in AF tissues. The custom diffusion apparatus (Fig. 2a), previously described,^{20,46} consisted of two solution chambers separated by a specimen holder in the middle. The specimen was held between two porous plates and sealed with an o-ring on the lateral edge. Uniaxial confined compression was applied by changing the metal spacers between the chambers. For oxygen experiments, the downstream chamber was equipped with an oxygen probe and sealed from the atmosphere (Fig. 2a).

At the start of the experiment, the specimen was confined to ~0% compression, calculated based on the initial and compressed heights of the tissue specimen. At this time, the upstream chamber was filled with a high concentration solution (e.g., 20 g/L glucose in PBS, or air-saturated PBS) while the downstream chamber was filled with regular PBS (for glucose tests) or deoxygenated PBS (for oxygen tests). Both chambers were stirred continuously using micro-magnetic stir bars. The change in concentration downstream was monitored over time. For glucose experiments, the downstream chamber was emptied at 15-minute intervals and the glucose concentration was measured using a glucose assay (Sigma-Aldrich, Inc., St. Louis, MO) and spectrophotometer (SmartSpecTM Plus Spectrophotometer, Bio-Rad Laboratories, Inc., Hercules, CA). The experiment was continued until steady-state was reached (i.e., the same (within 5%) measured for 2–3 consecutive intervals). For oxygen experiments, the oxygen concentration downstream was measured continuously for one hour using an optical oxygen sensor (Ocean Optics, Inc., Dunedin, FL). The apparent diffusion coefficient, D , was calculated based on Fick's Law and conservation of mass^{20,25,46}:

$$D = \ln \left[\frac{C_{up} - C_{down}(t_o)}{C_{up} - C_{down}(t)} \right] \frac{V_{down} h}{A(t_o - t)}, \quad (1)$$

where $C_{down}(t_0)$ is the concentration of solute in the downstream chamber at time t_0 (initial time) and $C_{down}(t)$ is that at time t , C_{up} is the concentration of solute upstream, V_{down} is the volume of solution downstream, h is the height (or thickness) of the specimen, and A is the cross-sectional area through which the solute diffuses. The apparent diffusion coefficient calculated here is related to the effective diffusivity, D_{eff} , by a factor of the partition coefficient, K : $D = KD_{eff}$; for oxygen, $D = D_{eff}$ since it is assumed that $K = 1$.

Glucose Partition Coefficient Measurement

The glucose partition coefficient, or solubility, was measured based on a previously described method in the literature.^{11,37} The concentration of a solute in a tissue at equilibrium, c , is related to that in the bathing solution, c^* , by: $c = Kc^*$. The partition coefficient can be measured by equilibrating a tissue in a sequence of two baths, one with, and one without solute. By conservation of solute mass between the two baths, the partition coefficient is calculated by^{11,37}:

$$K = \frac{V_{b2}c_2}{\phi^w V_t(c_1 - c_2)} \quad (2)$$

where V_{b2} is the volume of the second equilibration bath, ϕ^w is the tissue porosity, V_t is the tissue volume, and c_1 and c_2 are the equilibrium solute concentrations in baths 1 and 2, respectively.

A custom-designed chamber was used to measure the partition coefficient of glucose in AF tissue (Fig. 2b). The chamber allowed for confinement and sustained tissue compression during the equilibration period. The specimen was first equilibrated in 100 μ L of high glucose (5 g/L) Dulbecco's Modified Eagle's Medium (DMEM) at 4°C for 24 hours. The equilibrium glucose concentration was measured using a commercially available glucose meter (Accu-Check Aviva, Roche Diagnostics, Inc.) at the end of the 24 hour period. The specimen was then equilibrated in three consecutive baths of 100 μ L of DMEM containing no glucose at 2°C for 8 hours each, for a total of 24 hours. The contents of the three equilibrium baths were combined and mixed, and the glucose concentration was measured. All equilibration baths were stirred constantly using a micro-stir bar throughout the equilibration period.

Tissue Porosity Measurement—A buoyancy method was used to measure the water volume fraction, or porosity, of tissue specimens.¹³ The porosity of the sample in the uncompressed state, ϕ_o^w , was calculated based on the specimen weight in air, W_{wet} , the weight in phosphate buffered saline (PBS) solution, W_{PBS} , and the dry weight after lyophilization, W_{dry} :

$$\phi_o^w = \frac{(W_{wet} - W_{dry}) \rho_{PBS}}{(W_{wet} - W_{PBS}) \rho_w}, \quad (3)$$

where ρ_{PBS} and ρ_w are the densities of PBS solution and water, respectively. Because specimens for diffusivity experiments were used for three tests (at three levels of strain), the porosity in the deformed tissue was estimated using the relationship between tissue water content and tissue dilatation, e (i.e., relative volume change, which is related tissue deformation, J , by: $e = J - 1$);^{20,24}

$$\phi^w = \frac{\phi_o^w + e}{1 + e}, \quad (4)$$

where ϕ^w is the tissue porosity of the compressed tissue. Given that the tissues were under uniaxial confined compression for these experiments, the level of axial strain was taken to be the same as the volumetric strain (or dilatation).

RESULTS

The results for diffusivity of glucose and oxygen at room temperature ($23.6 \pm 1.4^\circ\text{C}$) in human AF tissue can be found in Table 1. For each sample, the value of compressive strain was calculated based on the initial and compressed heights of the specimen. The values for the glucose diffusion coefficient ranged from $3.56 \times 10^{-7} \text{ cm}^2/\text{s}$ to $8.71 \times 10^{-7} \text{ cm}^2/\text{s}$ for all samples. Two-way ANOVA analysis of variance (SPSS 11.5, SPSS, Inc., Chicago, IL) indicated that glucose diffusivity was significantly affected by both compression and direction of diffusion ($p < 0.05$). Tukey post-hoc analysis showed that all strain groups were significantly different for all three directions of diffusion (i.e., $0\% > 10\% > 20\%$). Furthermore, radial diffusion was significantly lower than that in the axial or circumferential directions, indicating that glucose diffusivity in human AF is anisotropic. Glucose diffusivity in the axial and circumferential directions did not differ significantly at any level of strain.

The range of values for the oxygen diffusion coefficient was $1.13 \times 10^{-6} \text{ cm}^2/\text{s}$ to $1.85 \times 10^{-5} \text{ cm}^2/\text{s}$ for all samples. For oxygen diffusivity, two-way ANOVA did not show a statistical significance ($p = 0.05$), although one-way ANOVA indicated that compression significantly decreased diffusivity for the circumferential group ($p < 0.05$). Moreover, student's t-tests indicated that radial diffusivity was significantly lower than circumferential diffusivity at 0% and 10% compressive strains ($p < 0.05$).

The results for glucose partition coefficient in human AF tissue are shown in Fig. 3. For all specimens, the glucose partition coefficient was found to be 0.82 ± 0.054 at $1.45 \pm 0.9\%$ compressive strain ($n = 12$), 0.76 ± 0.044 at $13.2 \pm 1.2\%$ ($n = 12$) compressive strain and 0.71 ± 0.076 at $26.4 \pm 1.9\%$ compressive strain ($n = 12$). The compressive strain was calculated based on the initial thickness of the specimen and the compressed thickness (2 mm for all specimens). One-way ANOVA showed a significant decrease in the partition coefficient with increase in compression ($p < 0.05$). Tukey post-hoc analysis indicated that only the partition coefficients at 0% and 20% nominal strain levels differed significantly.

The effective diffusion coefficient, D_{eff} , of glucose was calculated based on the results for apparent diffusivity, D , and partition coefficient, K . A linear curve-fit of the results for glucose partition coefficient versus tissue porosity was performed, see Fig. 3. The effective diffusivity was calculated for each specimen at each level of compression based on the porosity of the compressed tissue using the relationship $D = KD_{eff}$ and the correlation shown in Fig. 3. These results are also shown in Table 1; the values for effective diffusivity of glucose in human AF ranged from $5.36 \times 10^{-7} \text{ cm}^2/\text{s}$ to $1.09 \times 10^{-6} \text{ cm}^2/\text{s}$ for all samples. For oxygen, the apparent diffusivity is equal to the effective diffusivity, based on the assumption that the partition coefficient for oxygen in AF is unity.

DISCUSSION

To our knowledge, this is the first study to investigate the strain-dependent and anisotropic behaviors of nutrient transport in human IVD tissues. Values found here for effective diffusion coefficients of oxygen and glucose in human AF, ranging from $6.62 \times 10^{-7} \text{ cm}^2/\text{s}$ to $9.77 \times 10^{-6} \text{ cm}^2/\text{s}$, are within the range of values for these solutes in animal and human IVD found in the literature.^{20,29,34,46} The values of glucose partition coefficient in human AF tissue, ranging from 0.71 to 0.82, are similar to previously reported values in

cartilaginous tissues, which range from 0.67 to 0.9.^{27,29,40} Furthermore, the general trends of decreased transport with increasing compressive strain, as well as direction-dependent transport, are also in agreement with results in the literature for various solutes in cartilaginous and IVD tissues, see reviews.^{19,41} The inverse relationship between solute size and the diffusion coefficient, with oxygen diffusivity being higher than glucose diffusivity, is also consistent with previous findings; see review.¹⁹

The strain-dependent behavior of nutrient diffusion and partitioning in AF tissue is likely the result of fluid exudation during tissue deformation. That is, when the tissue is compressed, water flows out and tissue porosity (or water content) as well as pore size decrease. Our results are in agreement with previous studies on solute transport in compressed IVD tissues.^{9,10,20,46} For glucose, the application of strain significantly affected diffusivity in all directions as well as solute partitioning in human AF tissue. The results for oxygen diffusivity showed the same trend, although no statistical significance was found for axial and radial groups, likely due to the small sample size. Further, the difference in compression effects on these two solutes may result from the difference in the sizes of oxygen and glucose molecules (i.e., Stokes' radius of glucose, 0.36 nm, compared with 0.10 nm for oxygen). A previous study showed that compression more strongly affected transport of larger molecules in cartilage.³⁷

The range of compressive strain used in this study is similar to the range of those seen in IVD *in vivo*^{12,33,45}; although, it should be noted that the compressive strains in this study were local strain values for the tissue specimens and may not represent the precise *in vivo* conditions. Nonetheless, since disc cells rely on transport to receive an adequate supply of oxygen and glucose to maintain viability and functioning, understanding the factors affecting nutrient transport in the IVD is important for better elucidating nutritional supply in the tissue and related disc degeneration. The reduced transport of nutrients under deformation levels similar to those in the *in vivo* environment indicates that compression occurring during normal daily activity may hinder the availability of nutrients in the disc, which can be detrimental to disc cells. In addition to the effects on transport properties, mechanical loading may also result in changes in disc cellular metabolism, either due to an alteration in intrinsic cellular activity or because of changes in the extracellular environment (i.e., changes in matrix composition and/or nutritional levels following compression). These effects should be further investigated in order to more clearly understand how mechanical loading influences nutritional supply and cellular activity in the IVD.

Although the strain applied to specimens in these experiments can be considered as “local” strain, as discussed above, this information can be included in a mathematical model of the IVD, in which local strain values are used to calculate solute concentrations in the tissue. Therefore, the quantitative data provided by this investigation is essential to the development of accurate numerical models to predict the *in vivo* nutritional environment in the disc.

Diffusivity of glucose in human AF was found to be anisotropic, being lower in the radial direction as compared to the axial and circumferential directions. A similar trend was found for oxygen diffusivity, although results were not statistically significant. The complex structure and organization in AF tissue likely plays a primary role in the anisotropic nature of solute diffusivity in the tissue. As noted, the AF has a highly organized, layered structure, as well as “microtubes” extending along the collagen fiber direction within the lamellae, and visible in both axial and circumferential specimens, but not in radial specimens.^{18,20,41} It is thought that these microtubes may facilitate, or provide a preferred route for, transport in the axial and circumferential directions; that is, solutes may diffuse through the microtubes without significant hindrance by the collagen fiber network. In contrast, in radial specimens,

the microtubes are not visible, nor do they appear to be contiguous between adjacent lamellae, so solutes diffuse through the dense ECM with greater impediment, thereby resulting in a lower diffusion coefficient in the radial direction.

The difference in results for anisotropic diffusion of oxygen and glucose in human AF may again be attributed to difference in sizes of these molecules. Several previous investigators have reported on the direction-dependent diffusivity in IVD, see a recent review.⁴¹ It has been suggested that radial hindrance to diffusion caused by collagen fiber organization increases with increasing solute size.⁴¹ The results in this investigation further confirm this notion.

These findings are significant given the transport routes in the IVD *in vivo*. Studies have shown that the disc receives its nutrition either axially, via the endplate route, or radially, via the periannular route. The majority of *in vivo* (using animal model) and *in vitro* studies have suggested that the endplate route is the main pathway for exchange of solutes between the NP (and the inner AF) and the surrounding vasculature.^{6,7,15,29,30,35,43} However, during degeneration and aging, calcification of the cartilage endplates is common, and is believed to lead to decreased permeability to nutrients. On the other hand, Rodriguez et al recently showed that the blood supply at the endplate is reduced for degenerated IVD (i.e., reduced quality of capillaries in the vertebral bodies), which contributes to the poor nutritional supply to disc cells.³⁸ In either case, nutrients must be supplied from the blood vessels at the annular edge of the disc, moving to the center of the disc in the radial direction. As our results indicate, radial diffusion is slower, likely due to the lack of microtube structure in the tissue in the radial direction, and therefore nutrition to the disc center may be further impaired, thereby accelerating the degeneration cascade.

Several previous studies investigating the diffusivity of water in normal and degenerated IVD have reported that diffusivity decreased with increased degeneration.^{4,21,23,32} Disc degeneration is most notably marked by loss of tissue water content, contributing to the decreased transport rates in the tissue. In this study, only four discs were obtained for tissue extraction, with only two discs being considered as “degenerated” (grades II and III). Therefore, it is difficult to deduce overall relationships between nutrient transport and degeneration based on these results.

There are several limitations of the current study that should be noted. Due to difficulty in obtaining human IVD, only four discs were used in glucose studies, and three for oxygen studies. In order to more fully understand how factors such as diffusion direction, tissue compression and degenerative grade affect transport in the IVD, tissues from a larger number of discs should be investigated in a future study. Additionally, the use of porous plates to secure and compress the tissue samples in diffusion experiments may result in stagnant layer formation at the tissue boundary, impacting the measurements for diffusivity. The use of a stirring rod has been shown to minimize boundary layer formation²⁸; however, it is likely that it could not be eliminated entirely. Previous studies have shown that porous plate may cause a 7% underestimation of the diffusion coefficient measurement.²⁰

One further limitation in this study is that experiments were carried out at room temperature ($23.6 \pm 1.4^\circ\text{C}$), rather than at the higher body temperature (37°C). Since diffusion is a temperature-dependent process, the data shown are not directly reflective of the *in vivo* situation. Using the Stokes-Einstein equation, the values obtained in this study can be easily converted to those at body temperature. Alternatively, the diffusivity can be expressed in terms of relative diffusivity, D/D_o , where D_o is the diffusivity of the solute in aqueous solution at the same temperature (e.g., for glucose at $\sim 24^\circ\text{C}$, $D_o \sim 6.4 \times 10^{-6} \text{ cm}^2/\text{sec}$, while $D_o \sim 2.5 \times 10^{-5} \text{ cm}^2/\text{sec}$ for oxygen at $\sim 24^\circ\text{C}$).

In summary, we have reported, to our knowledge, the first quantitative results for anisotropic and strain-dependent nutrient diffusivity and partitioning in human annulus fibrosus tissue. Our findings show that glucose diffusivity in human AF follows a significant strain-dependent and anisotropic behavior. While the general pattern of behavior was the same for oxygen diffusivity in the tissue, there were not statistically significant differences between all groups. We also found that glucose partitioning (solubility) in human AF is strain-dependent. The results of this investigation are important for better understanding the nutritional transport pathways and mechanisms in the intervertebral disc. Furthermore, this information will add important insights into the relationship between nutritional supply and degenerative changes to the intervertebral disc, thereby elucidating the pathophysiology of low back pain.

Acknowledgments

This study was supported by grants from NIH (AR050609, AR056101 and EB008653) and an NRSA Fellowship from NIH NIA (AG030921). The authors wish to thank Mr. Andre Castillo, BSBE, for his help in apparatus development.

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Figure 1.

Schematic diagram showing the structure of the IVD as well as the size and orientation of AF specimens for diffusivity and partitioning experiments. This diagram does not represent the specific locations within the AF region from which specimens were harvested for the particular experiments; for all experiments, specimens were taken from both the anterior and posterior AF.

(a)

(b)

Figure 2.

Schematic diagram of the custom-designed chambers for measuring (a) diffusivity and (b) partition coefficient. For oxygen measurements, the diffusivity chamber includes an optical oxygen sensor and air-tight sealing cap, as shown in (a); this sensor is not present for glucose diffusivity measurements. The metal spacer between the two chamber halves is used to control the amount of uniaxial confined compression on the specimen; that is, the spacer matches the desired compressed height of the tissue (i.e., for a 0.5 mm thick specimen at 0% strain, the spacer is 0.5 mm, while at 10% strain the spacer is changed to a 0.45 mm thickness).

Figure 3.

Results for glucose partition coefficient, K , versus tissue porosity in human AF tissue. Results shown are pooled data from both axial and circumferential directions at all three strain levels. There was no statistical difference between these two groups (i.e., partitioning is isotropic) in preliminary studies (data not shown), so no distinction was made between tissue orientations. Tissue porosity was directly calculated using the described buoyancy method.

Results for Glucose and Oxygen Diffusivity in Human Annulus Fibrosus in Three Principal Directions and At Three Levels of Compressive Strain

Table 1

	Glucose				Oxygen			
	<i>n</i>	Strain (%)	<i>D</i> (×10 ⁻⁷ cm ² /s)	<i>D_{eff}</i> (×10 ⁻⁷ cm ² /s)	<i>n</i>	Strain (%)	<i>D</i> = <i>D_{eff}</i> (×10 ⁻⁶ cm ² /s)	
Axial	16	3.06 ± 3.09	7.56 ± 0.75	9.41 ± 1.00	12	0.46 ± 0.62	8.53 ± 4.35	
	16	12.8 ± 2.78	6.52 ± 0.65	8.44 ± 0.89	12	10.1 ± 0.94	7.14 ± 3.74	
	16	22.4 ± 2.47	5.74 ± 0.72	8.24 ± 1.13	12	19.7 ± 1.80	5.62 ± 4.56	
Circumferential	16	4.97 ± 4.25	7.40 ± 1.06	9.29 ± 1.04	12	0.33 ± 0.67	9.77 ± 3.75	
	16	14.5 ± 3.83	6.32 ± 0.81	8.34 ± 0.88	12	9.31 ± 1.52	6.81 ± 1.43	
	16	24.0 ± 3.40	5.38 ± 0.76	8.01 ± 1.04	12	19.5 ± 1.86	4.93 ± 1.97	
Radial	16	2.43 ± 2.35	5.85 ± 0.27	7.44 ± 0.42	12	0.69 ± 0.55	6.45 ± 3.31	
	16	12.2 ± 2.12	5.18 ± 0.35	6.90 ± 0.63	12	9.70 ± 1.06	5.09 ± 2.66	
	16	21.9 ± 1.88	4.46 ± 0.43	6.62 ± 0.89	12	19.5 ± 1.32	4.40 ± 2.41	