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Mass transport kinetics of the DNA-binding dye Hoechst-33342 into bovine spermatozoa

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Abstract—The mass transport kinetics for the unmediated diffusion of Hoechst-33342 dye (HO-33342) into spermatozoa was established at varying pH and temperature. The mass transport diffusion coefficient k_{diff} at 41.5 °C and pH 6.2 was determined to be 0.0435 min^{-1} [M sperm/mL]$^{-1}$ and the energy of activation E_a associated with the temperature dependency of k_{diff} at 14.2 kcal/gmoL. The relationship of k_{diff} to pH was based mechanistically on the premise that the HO-33342 dye becomes more hydrophobic as the pH becomes more alkaline and therefore facilitates transport. The k_{diff} dependency on pH was described mathematically using a form of the Henderson–Hasselbalch equation in which the pK_a of the HO-33342 dye was 6.6. Using the kinetic parameters, an Excel-based model was developed capable of predicting the time required for the HO-33342 dye to reach any specified level of saturation in the spermatozoa.

1. Introduction

Introduction of fluorescent dyes into living cells is of significant importance in many areas of biology. For technology directed at the separation of X chromosome-bearing spermatozoa from their Y chromosome counterparts,[1] an essential component to this technology is the introduction of the DNA-binding dye, Hoechst-33342, which has been shown to be valuable in discriminating between the X and Y bearing spermatozoa of several mammalian species such as rabbit, boar, ram, and bull through the use of flow cytometry.[2] The Hoechst-33342 (HO-33342) dye is a bis-benzimidazole that binds to DNA.[3] The excitation of the HO-33342-DNA complex with UV–vis radiation at 350 nm wavelength provides an emission spectrum with maximum wavelength at 460 nm.[4] Since the equilibrium coefficient K_a of HO-33342 to calf thymus DNA[3] was measured at 2.8×10^7 M^{-1}, the total amount of dye in the sperm cell will essentially be bound to the DNA. Therefore, the intensity signal from the emission spectra can be considered to be directly proportional to the concentration of the DNA-dye complex which must reach a critical level for detecting differences in signal intensity between the X and Y chromosomes. Furthermore, due to this propensity for the HO-33342 dye to bind to DNA, the rate determining step in forming the dye-DNA complex would involve the transport of the dye across the sperm cell membrane. Precedent in the literature has suggested that HO-33342 enters the cell by an unmediated diffusion transport mechanism through the cell membrane prior to DNA binding.[5] However, there appears to be a paucity of information regarding the detailed study of the mass transport kinetics associated with this mechanism. There is value in determining the kinetics since this information could be used to establish a model for predicting the length of time needed for incubating the cells with HO-33342 dye to achieve the necessary fluorescence intensity for X/Y discrimination. This paper discusses the kinetic study pursued in our laboratory and the utilization of the kinetic parameters in developing the predictive model.

2. Results and discussion

Based on precedent[5] the unmediated diffusion mechanism of HO-33342 through the spermatozoa membrane was used as the premise for establishing the mass transport kinetics. Fluorescence spectroscopy was used to measure the rate of dye uptake into the cell. Shown in Figure 1 are the emission spectra for the HO-33342-DNA complex in spermatozoa obtained at varying time

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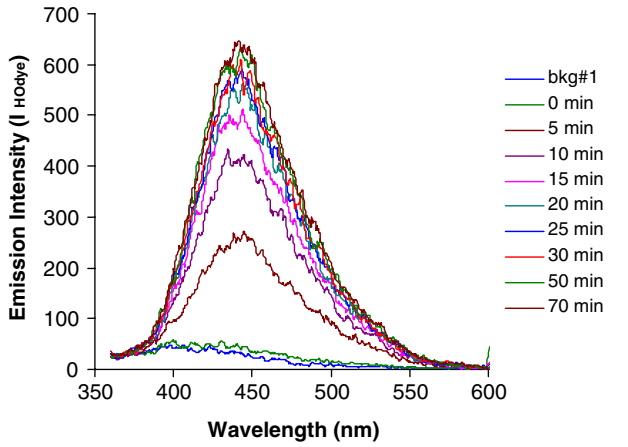

Figure 1. Emission intensity versus wavelength data was obtained on a 3 Million/mL spermatozoa suspension in citric acid monohydrate/$KHCO_3$/$NaHCO_3$ buffer using the Perkin-Elmer LS55 Luminescence Spectrometer at pH 7.0 and 41.5 °C. Concentration of the HO33342 dye was 209 nM.

intervals over the course of 70 min whereby the maximum peak emission intensity was approached at 30 min indicating sperm cell saturation. The experiment was conducted in a citrate/bicarbonate-based buffer at 41.5 °C and pH 7.0. The cells were excited at 350 nm wavelength and the emission spectra were acquired in the 360–600 nm wavelength interval.

The experimentally observed maximum peak intensities at 440 nm wavelength were plotted as a function of time (Fig. 2). The diffusion rate represented by the increase in emission intensity with time was greatest during the first 15 min after dye addition to the sperm cell suspension. As time increased, the intensity increase rate decreased until finally approaching a rate of zero which was likely due to the saturation level of the dye in the cell. This observed behavior of dye diffusion into the sperm cells suggested 1st-order diffusion kinetics in which the rate is proportional to the difference in concentration of the dye at any given time and the concentration at saturation levels.

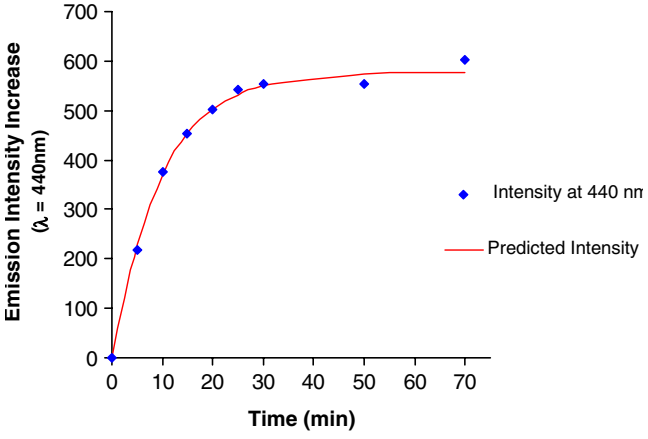

Figure 2. Emission intensity versus time experimental data (diamonds) were obtained from the data shown in Figure 1 along with the curve fit to the data based on 1st-order diffusion kinetics.

Prior to further discussion, the nature of the ejaculates used in this study should be mentioned. The spermatozoa contained in the ejaculate consisted of two subpopulations, live and dead cells. Since dead cells would not have the ability to control the movement of the dye across the membranes, the diffusion rate was expected to be very rapid. Although the live cell subpopulation was expected to have subtle differences in their membrane morphologies, the emission intensity data were assumed to represent an average rate for all live cells. Therefore, the live cell population was used in the validation process of determining whether the diffusion of the HO33342 dye into the sperm cells was 1st-order. The difference between the rates for the live and dead cell subpopulations was noted in the emission intensity versus time data for a greater portion of the experiments in that the rate of increase in the emission intensity was significantly greater between initial time ($t = 0$) and the next data point (acquired between 30 s and 5 min) than for the remaining data points at subsequent time intervals. Therefore, for those experiments exhibiting this behavior, treatment of the data for validating the 1st-order diffusion kinetics was performed by using all of the emission intensity data except for the value at initial time.

The 1st-order mass transfer rate is represented by Eq. 1.[6] The proportionality constant between the rate and the concentration gradient between saturated and unsaturated HO-33342 dye concentration levels is the mass transfer rate coefficient k_{obs}. The bridge between the rate expression represented by Eq. 1 and the experimental data is depicted as Eq. 2. Eq. 2 is essentially the same as Eq. 1 except the concentration terms were replaced by emission intensity levels.

Mass transfer rate equation

$$\text{Rate} = \frac{dC_{\text{HOdye}}}{dt} = k_{\text{Obs}} * (C_{\text{HOdye-sat}} - C_{\text{HOdye}}) \quad (1)$$

Mass transfer rate equation—emission intensity

$$\frac{d(I_{\text{HOdye}})}{dt} = k_{\text{Obs}} * (I_{\text{HODye-sat}} - I_{\text{HOdye}}) \quad (2)$$

The first step toward obtaining the 1st-order kinetic data and k_{obs} was to integrate Eq. 2. The integrated rate expressions are represented by Eqs. 3 and 4 in exponential and logarithmic forms, respectively.

Integrated mass transfer rate equation—emission intensity

$$I_{\text{HOdye}} = I_{\text{HOdye-sat}} * [1 - e^{-k_{\text{Obs}} * t}] \quad (3)$$

$$\ln[(I_{\text{HOdye-sat}} - I_{\text{HOdye}})/I_{\text{HOdye-sat}}] = -k_{\text{Obs}} * t \quad (4)$$

The proposed 1st-order kinetic mass transfer rate of HO-33342 into the spermatozoa was confirmed by fitting the integrated form of the rate expression (Eq. 3) to the experimental data shown in Figure 2 by manually

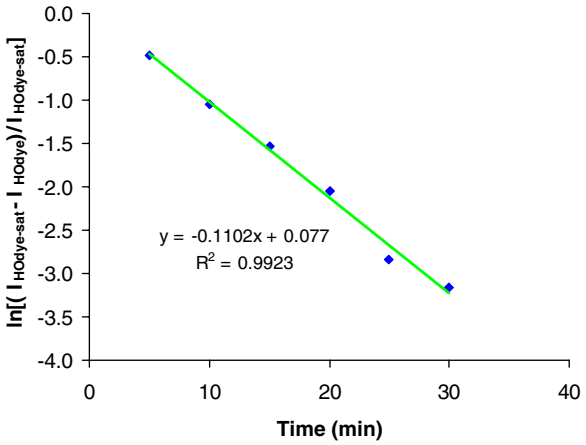

Figure 3. Observed kinetic rate coefficient (k_{obs}, min^{-1}) obtained by logarithmic plot of the emission intensity versus time.

adjusting the k_{obs} value. From this curve fitting approach, the emission intensity at HO-33342 dye saturation in the sperm cell $I_{HOdye\text{-}sat}$ was obtained. Further confirmation of the 1st-order kinetics was established by obtaining a linear plot of the left side of Eq. 4 versus time (Fig. 3). The absolute value of the slope of the best fit to the data provided the rate coefficient k_{obs}.

The mathematical treatment of the 1st-order kinetics of HO-33342 dye mass transport is completed with the presentation of Eqs. 5–7. Eq. 5 represents the transformation of the integrated emission intensity 1st-order rate equation (Eq. 4) back to concentration terms. The rate coefficient k_{obs} was normalized with respect to sperm concentration and % motility (Eq. 6) to produce k_{diff}. The parameter k_{diff} therefore reflects the kinetic data acquired for a specified unit of sperm concentration (M sperm/mL) and for live spermatozoa (%motility). Finally, the time required to transport the HO-33342 dye into the sperm cells at a given percentage of the dye-saturated concentration level was obtained by using Eq. 7. Specifically Eq. 7 was obtained by manipulating Eq. 5 to obtain time as a function of the dye concentration and k_{diff} with the unsaturated dye concentrations represented as a fraction of the saturated dye concentration.

Integrated mass transfer rate equations—dye concentration

$$\ln[(C_{HOdye\text{-}sat} - C_{HOdye})/C_{HOdye\text{-}sat}] = -k^{*}_{diff}t \quad (5)$$

$$k_{diff} = \frac{k_{obs}}{[\text{Sperm Conc.}] * (\%\text{Motility}^{*}.01)} \quad (6)$$

$$t = \frac{-\ln[C_{HOdye\text{-}sat} * (1-n)/C_{HOdy\text{-}sat}]}{k_{diff}} \quad (7)$$

C_{HOdye} $n * C_{HOdye\text{-}sat}$ n = fraction of $C_{HOdye\text{-}sat}$

[Sperm Conc.] = Million/mL

The experimental determination of k_{obs} and k_{diff} at varying temperature and pH is shown in Table 1. Since the quality of semen samples varies considerably between ejaculates (%motility) due to environmental factors such as seasonal temperature variance throughout the calendar year,[7] experiments designed to compare differences in pH and temperature were performed on the same ejaculate on the same day. As an example, experiments were performed on bull # 7963 ejaculate at pH 6.26 and 7.45 (entries 3 and 4) on the same day (5/26/2006). This protocol was critical in that significant variance of k_{diff} values was noted for different ejaculates of the same bull (#7963) at essentially the same pH and temperature (compare entries 12 and 14 and entries 15 and 16).

Table 1. Results of kinetic experiments: diffusion of Hoechst-33342 dye into spermatozoa

Entry (Date)	Bull ID[a]	Temperature (°C)	pH	% Motility	k_{obs}[b]	k_{diff}[c]
1 (5/19/06)	7963	41.5	7.04	76	0.1102	0.0483
2 (5/19/06)	7963	41.5	7.15	76	0.1427	0.0626
3 (5/26/06)	7963	41.5	6.26	76	0.0307	0.0135
4 (5/26/06)	7963	41.5	7.45	76	0.1411	0.0619
5 (6/12/06)	8519	41.5	6.21	90	0.1056	0.0391
6 (6/12/06)	8519	41.5	7.23	77	0.2404	0.1041
7 (6/26/06)	8519	41.5	6.25	68	0.2229	0.1093
8 (6/26/06)	8519	41.5	7.71	66	0.3260	0.1646
9 (8/10/06)	8519	41.5	7.36	37	0.2831	0.2550
10 (8/23/06)	8519	41.5	6.21	68	0.0245	0.0120
11 (8/23/06)	8519	41.5	7.63	64	0.2176	0.1133
12 (6/15/06)	7963	32.0	6.22	80	0.0375	0.0156
13 (6/15/06)	7963	32.0	7.18	80	0.0673	0.0280
14 (7/17/06)	7963	32.0	6.17	82	0.0181	0.0074
15 (7/17/06)	7963	32.0	6.82	84	0.0238	0.0094
16 (8/01/06)	7963	32.0	6.86	55	0.0436	0.0264
17 (8/01/06)	7963	32.0	7.84	55	0.1553	0.0941
18 (8/10/06)	8519	32.0	7.32	43	0.1622	0.1257
19 (7/06/06)	8519	36.9	6.23	85	0.0212	0.0083
20 (7/06/06)	8519	36.9	7.16	83	0.0450	0.0181

[a] Mason–Plunkett farms (Bonne Terre, MO, USA).
[b] Mass transfer rate coefficient k_{obs} (min^{-1}) obtained from the slope of the emission intensity versus time graph (Fig. 4) generated from using Eq. 4.
[c] Mass transfer coefficient k_{diff} (min^{-1}(M sperm/mL)$^{-1}$) obtained by transforming k_{obs} (min^{-1}) using Eq. 6. Sperm concentration units are in Million/mL.

From the data presented in Table 1, significant trends were noted. The k_{obs} and k_{diff} values were for the most part greater at 41.5 °C (entries 1–11) than at 32 °C (entries 12–18) and 36.9 °C (entries 19–20). Likewise, for a specific temperature, k_{obs} and consequently k_{diff} tended to be greater at more alkaline pH. This result especially was noted when two experiments at two different pH values were performed on the same ejaculate and on the same day. It was encouraging to have observed these trends given that sperm quality (%motility) varied significantly between ejaculates.

Based on these trends, average values for the k_{diff} parameters were obtained by averaging the data presented in Table 1 for a given pH and temperature (Table 2). As an example, at pH 6.2 and 41.5 °C, the average k_{diff} parameter was determined using the data from Table 1: entries 3, 5, 7, and 10. Additional average values for k_{diff} at 41.5 °C were obtained at pH 7.2 and 7.6 and at pH 6.2, 6.8, and 7.2 for 32.0 °C.

The next step in establishing the mass transport kinetics of HO-33342 dye into spermatozoa was to determine the impact of temperature on the k_{diff} parameter. The Arrhenius equation[8] (Eq. 8) was used to describe this relationship. The reference value $k_{diff\ T(ref)}$ (Table 2: entry 1) used in Eq. 8 was the average k_{diff} obtained at pH 6.2 and 41.5 °C (reference temperature, T_{ref}). Using the data from two experiments performed on the same ejaculate at 32.0 and 41.5 °C (Table 1: entries 9 and 18), the energy of activation E_a was determined to be 14.2 kcal/gmoL using Eq. (8a) (SI). With the $k_{diff\ T(ref)}$ and E_a values determined, the mass transport rate coefficient k_{diff} can be determined for any given temperature. The parameters necessary to perform this calculation are presented in Table 3 (entries 1–3).

$$k_{diff}(T) = k_{diff}T(ref) * e^{-E_{act}/R*[1/T(^{\circ}K)-1/T_{ref}(^{\circ}K)]}$$
$$T_{ref}(^{\circ}C) = 41.5\ ^{\circ}C \qquad (8)$$

Determining the impact of pH on the k_{diff} value required a proposed mechanism for the mass transport process.[9] The HO-33342 bis-benzimidazole dye at pH 6.2 is likely a dication in which one of the imidazole groups and at the piperazine moiety is protonated.[10] Increasing the pH in the spermatozoa suspension should convert a significant portion of the di-cationic dye into the mono-cationic form thereby rendering the compound more hydrophobic which would enable the species to pass through the hydrophobic membrane more readily. Although the mechanism of HO-33342 transport is highly complex involving the presence of transporter proteins and ATP,[4,11] a simplistic mechanism of the HO-33342 transfer across the membrane as a result of increased hydrophobicity at higher pH is presented in Figure 4.

Table 2. Diffusion of Hoechst-33342 dye into spermatozoa—kinetic parameters

Entry	Experimental k_{diff}[a]	pH	Temperature (°C)	Predicted k_{diff}[h]
1	0.0435[b]	6.2	41.5	0.0435
2	0.1220[c]	7.2	41.5	0.1221
3	0.1390[d]	7.6	41.5	0.1388
4	0.0115[e]	6.2	32.0	0.0214
5	0.0179[f]	6.8	32.0	0.0462
6	0.0769[g]	7.2	32.0	0.0601

[a] min^{-1}.
[b] Average of Table 1: entries 3, 5, 7, 10.
[c] Average of Table 1: entries 1, 2, 9.
[d] Average of Table 1: entries 8, 11.
[e] Average of Table 1: entries 12, 14.
[f] Average of Table 1: entries 15, 16.
[g] Average of Table 1: entries 13, 18.
[h] Determined from the HO33342 mass transport kinetic model (SI).

Table 3. Kinetic parameters associated with temperature and pH impact on k_{diff}

Entry	Kinetic parameter	Value	Unit
1	Reference k_{diff} (T = 41.5 °C, pH = 6.2)[a]	0.0435	min^{-1}(M sperm/mL)$^{-1}$
2	E_{act}[b]	14.2	kcal/gmoL
3	R[c]	0.001987	kcal/gmoL/°K
4	pK_a(dye)[d]	6.6	Dimensionless

[a] 314.5 °C = T_{ref}(°K) = 41.5 °C + 273 (Eq. 8).
[b] Energy of activation.
[c] Eq. 8a (SI).
[d] Eq. 9 (Refs. 9 and 11).

Based on the proposed transport mechanism, the relationship between k_{diff} and pH can be described by Eq. 9[12] in which the pH dependency of k_{diff} is a function of k_{diff} at pH 6.2 at any given temperature ($k_{diff\ (T,\ pH\ =\ 6.2)}$), p$K_a$ of the protonated imidazole functionality of the HO-33342 dye[10] (Table 3: entry 4), the hydrogen ion concentrations at pH 6.2 and at the pH of the spermatozoa suspension. Detailed derivation of Eq. 9 is in the Supporting Information (SI) (Eq. (9a)).

$$k_{diff(T,pH)} = k_{diff}(T, pH = 6.2) * \frac{(10^{-pK_a(dye)} + 10^{-6.2})}{(10^{-pK_a(dye)} + 10^{-pH})} \qquad (9)$$

Using the k_{diff} value obtained at 41.5 °C and pH 6.2 (Table 3: entry 1), Eqs. 8 and 9 can be used to determine $k_{diff\ (T,\ pH)}$ at any given temperature and pH.

Having developed the mass transport kinetics $k_{diff\ (T,\ pH)}$ for HO-33342 dye introduction into spermatozoa, the time required for the DNA of sperm cells to acquire a given % of saturation can be determined (Eq. 7). The ability to predict the time required to saturate the DNA with HO-33342 dye to the level necessary for sorting X/Y bearing sperm cells is important for maximizing the % of viable spermatozoa in the final product (Artificial Insemination Straws).[13] An Excel-based kinetic model (included in Supporting Information) was developed in which the input variables are temperature, pH, and degree of saturation for the HO-33342 dye. The output parameters are the predicted k_{diff} value and the time required to achieve the degree of saturation. For our studies, a saturation value of 85% was chosen. Using this value along with the 41.5 °C and pH 7.3 provided a predicted dye transport time which is consistent with experimental observations. Using the kinetic model, a comparison between the predicted and experimental k_{diff}

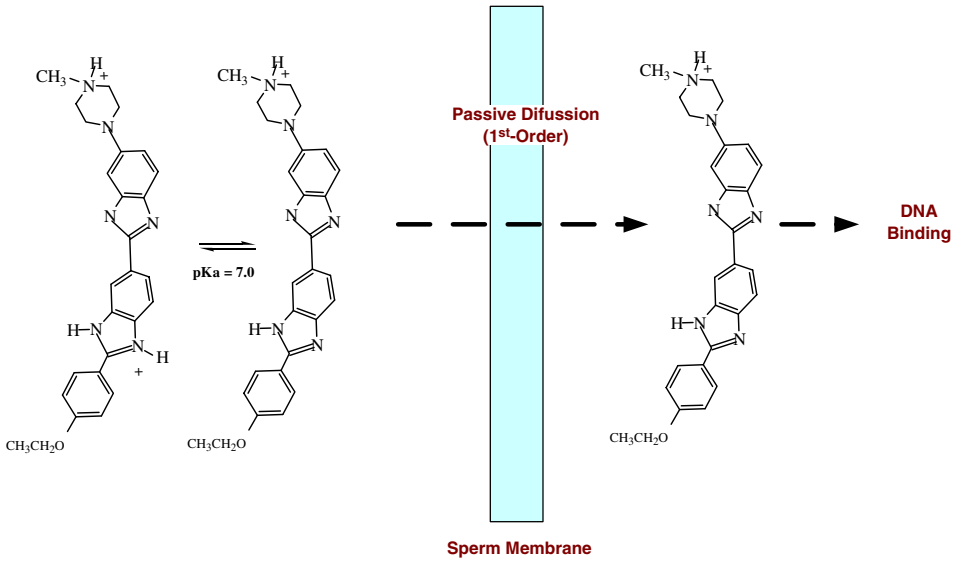

Figure 4. Hoechst-33342 dye unmediated diffusion (mass transfer) mechanism.

values at varying temperature and pH was made (Table 2: columns 2 and 5). At 41.5 °C, the comparison was excellent. However, differences between experimental and predicted k_{diff} were noted at 32.0 °C although the trends were consistently the same.

3. Conclusion

The mass transport kinetics for the unmediated diffusion of the Hoechst-33342 DNA-binding dye into spermatozoa was determined. The mass transport kinetic coefficient k_{diff} was obtained experimentally at varying temperature and pH. Due to the variance in the quality of ejaculate samples on different days, the critical aspect of the experimental protocol was that measurements on a given ejaculate comparing the effect of parameters such as pH and temperature must be performed on the same day. From the experimental data, the relationship between k_{diff} and temperature was determined using the Arrhenius relationship. The dependency of k_{diff} on pH was based mechanistically on the premise that the HO-33342 dye becomes more hydrophobic at higher pH and was therefore permitted to diffuse across the cell membrane. Finally, an Excel-based mathematical model was developed using the kinetic parameters and is capable of predicting the time required for the HO-33342 to reach a given saturation level within the spermatozoa. The establishment of the mass transport kinetics for the HO-33342 dye should be applicable in determining the unmediated diffusion rate of a variety of dyes into spermatozoa and other types of biological cells.

4. Experimental

4.1. Materials

Fluorescence spectra were obtained on a Perkin–Elmer LS55 Luminescence Spectrometer. The temperature in the LS55 cuvette holder was controlled by circulating water provided by the PolyScience Model 8006 Temperature Controller Circulating Water Bath. The Hoechst-33342 dye (1 mg/mL) was obtained from Invitrogen Co. (Carlsbad, CA). Sodium bicarbonate, potassium bicarbonate, and citric acid monohydrate were acquired from Sigma-Aldrich Co. (St. Louis, MO). Water used was GIBCO Ultrapure™ (Invitrogen Co.). Bovine ejaculates were supplied from the Plunkett-Mason Farms (Bonne Terre, MO). Sperm concentration (M Sperm/mL) and %motility were determined by IVOS (Integrated Visual Optical System—Hamilton Thorne Biosciences—Berverly, MA)[14,15] measurements prior to use in experiments. IVOS measurements were performed by incubating the spermatozoa in an 1.8 mL microcentrifuge tube for 10 min at 37 °C using a TCA-based buffer solution [citric acid monohydrate (1.77%), fructose (0.26%), pyruvic acid (0.10%), Trizma® base— 2-Amino-2-(hydroxymethyl)-1,3-propanediol (3.03%), GIBCO Ultrapure™ water (94.83%)] followed by injecting the spermatozoa into 4-chamber (20 μm) slides (Leja Corp—The Netherlands, info@leja.nl).

4.2. Experimental procedure

The buffer solution[16] was prepared by dissolving $NaHCO_3$ (0.661 g, 7.87 mmoL), $KHCO_3$ (1.380 g, 13.78 mmoL) in 40 mL of GIBCO Ultrapure™ water. Citric acid monohydrate (1.521 g, 7.239 mmoL) in 40 mL GIBCO Ultrapure™ water was slowly added to the bicarbonate solution over a 15 min time period to maintain pH at 6.0. The pH of the solution was noted to gradually increase with time due to CO_2 evolution. Neat bovine semen (7.7 μL, 1170 ± 44 M sperm/mL, 85 ± 0.6% motility) was added to a plastic cuvette. Semen sperm concentration and %motility were determined by IVOS. The buffer solution (3.0 mL) was SLOWLY added to the semen to prevent osmotic shock. The sperm concentration in the cuvette was 3.0 M sperm/mL. The cuvette was then placed in the LS55 Spectrometer cuvette holder equipped with water-jacket temperature control capabilities. The sample was

allowed to calibrate at 36.9 °C for 5 min. A background emission spectrum was acquired in the 350–600 nm wavelength range using a 350 nm excitation wavelength. After acquiring an additional background spectrum 5 min later, 6.3 μL of a 0.1 mM HO-33342 dye solution (0.07 nmoL HO-33342 dye/1 M sperm/mL) in water (GIBCO Ultrapure™) was quickly added to the sample. An initial time ($t = 0$ min) emission spectrum was then acquired at pH 6.20. Emission spectra were then acquired at 3, 6, 9, 15, 20, 30, 50, 70, 90, 110, and 125 min after HO-33342 dye addition. A pH measurement was recorded following each spectrum acquisition. During the course of the experiment, the pH increased to 6.27. The experiment was repeated on the same day using the same ejaculate sample except the buffer solution had been pre-treated with an aqueous KOH (3.95%)/NaOH (1.58%) solution to achieve a pH of 7.16.

4.3. Data evaluation

Using the FL WinLab software accompanying the LS55 Spectrometer, intensity data in 0.5 nm wavelength intervals from each of the emission spectra (350–600 nm range) were converted into Excel format. From these data, intensity versus wavelength plots were generated for the emission spectra at all time intervals. The peak intensities at 440 nm for the time intervals were then plotted against time to obtain a 1st-order exponential curve. The curve based on the algorithm (Eq. 3), $I_{\mathrm{HOdye}} = I_{\mathrm{HOdye\text{-}sat}}*\{1 - \exp(-k_{\mathrm{obs}}*t)\}$, was fit to the experimental data using Excel by manually adjusting the k_{obs} value. From this curve fitting exercise, the intensity at saturation of the HO-33342 dye into the spermatozoa ($I_{\mathrm{HOdye\text{-}sat}}$) was obtained. If the first attempt at curve fitting to the experimental data was not satisfactory, a reasonable curve fit was obtained by excluding the initial time point. After obtaining the saturation intensity value ($I_{\mathrm{HOdye\text{-}sat}}$), the observed mass transfer rate coefficient k_{obs} was obtained by plotting ($\ln[(I_{\mathrm{HOdye\text{-}sat}} - I_{\mathrm{HOdye}})/I_{\mathrm{HOdye\text{-}sat}}]$) versus time. The mass transfer coefficient k_{diff} normalized to live sperm concentration (M sperm/mL) was obtained by dividing k_{obs} by both the sperm concentration and fraction of live cells (%motility*.01) in the cuvette.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.06.061.

References and notes

1. (a) Garner, D. L. *Theriogenology* **2006**, *65*, 943–957; (b) Johnson, L.A.U.S. Patent 5,135,759, 1992.
2. (a) Johnson, L. A.; Welch, G. R. *Theriogenology* **1999**, *52*, 1323–1341; (b) Johnson, L. A. In *Agricultural Biotechnology*; Altman, A., Ed.; Marcel Dekker, Inc.: New York, 1998; pp 439–452; (c) Johnson, L. A. *Anim. Reprod. Sci.* **2000**, *60–61*, 93–107.
3. Harapanhalli, R. S.; McLaughlin, L. W.; Howell, R. W.; Rao, D. V.; Adelstein, S. J.; Kassis, A. I. *J. Med. Chem.* **1996**, *39*, 4804–4809.
4. Margolles, A.; Putman, M.; van Veen, H. W.; Konings, W. N. *Biochemistry* **1999**, *38*, 16298–16306.
5. LaLande, M. E.; Ling, V.; Miller, R. G. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 363–367.
6. Levenspiel, O. In *Chemical Reaction Engineering*, Second ed.; John Wiley & Sons: New York, 1972; pp 349–353.
7. (a) Nichi, M.; Bols, P. E. J.; Zuge, R. M.; Barnabe, V. H.; Goovaerts, I. G. F.; Barnabe, R. C.; Cortada, C. N. M. *Theriogenology* **2006**, *66*, 822–828; (b) Vogler, C. J.; Saacke, R. G.; Bame, J. H.; Dejarnette, J. M.; McGilliard, M. L. *J. Diary Sci.* **1991**, *74*, 3827–3835.
8. Price, N. C.; Dwek, R. A. In *Principles and Problems in Physical Chemistry for Biochemists*, Second ed.; Clarendon Press: Oxford, 1979; pp 153–157.
9. Eriksen, S. H.; Jensen, B.; Schneider, I.; Kaasgaard, S.; Olsen, J. *Appl. Microbiol. Biotechnol.* **1995**, *42*, 945–950.
10. Aleman, C.; Namba, A. M.; Casanovas, J. *J. Biomol. Struct. Dyn.* **2005**, *23*, 29–36.
11. Bart van den Berg van Saparoea, H.; Lubelski, J.; van Merkerk, R.; Mazurkiewicz, P. S.; Driessen, A. J. M. *Biochemistry* **2005**, *44*, 16931–16938.
12. Loudon, G. M. *J. Chem. Ed.* **1991**, *12*, 973–984.
13. Seidel, G. E., Jr.; Garner, D. L. *Reproduction* **2002**, *124*, 733–743.
14. Tardif, A. L.; Farrell, P. B.; Trouern-Trend, V.; Simkin, M. E.; Foote, R. H. *J. Androl.* **1998**, *19*, 201–206.
15. (a) Hamilton Thorne Biosciences; Technical Guide For IVOS, TOX IVOS, CEROS; Version 12.3; July 7, 2005, pp. 1–78; (b) Hamilton Thorne Biosciences; TOX IVOS Software Guide (HTM-TOX IVOS); Version 12.3, July 7, 2005, pp. 1–123; (c) Hamilton Thorne Biosciences Website: www.hamiltonthorne.com.
16. Salisbury, G. W.; Graves, C. N. *J. Reprod. Fertil.* **1963**, *6*, 351–359.