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Detection of hyaluronidase activity using fluorescein labeled hyaluronic acid and fluorescence correlation spectroscopy

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Abstract

The over-expression of hyaluronidase has been observed in many types of cancer, suggesting that it may have utility for diagnosis. Here we present a technique for the detection of hyaluronidase using Fluorescence Correlation Spectroscopy (FCS). Hyaluronan macromolecules (HAs) have been heavily labeled with fluorescein amine resulting in strong self-quenching. In the presence of hyaluronidase, HA is cleaved into smaller, fluorescein-labeled fragments and the self-quenching is released. Such cleavage is manifested by the increased average diffusion rate of the HA fragments, increased concentration of individual, fluorescent HA fragments, and increased intensity. All three of these properties are monitored simultaneously throughout FCS measurements, both as a function of time and hyaluronidase concentration. The method we present provides a sensitive measure of hyaluronidase activity and requires extremely small amounts of the HA substrate.

Keywords

Fluorescence Correlation Spectroscopy; Hyaluronan; Hyaluronidase; Fluorescence; Self-quenching

Introduction

The hyaluronidases (HA-ase) constitute a family of enzymes that degrade hyaluronan (HA), although chondroitin and chondroitin sulfate can be degraded at a significantly slower rate

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[1]. The hyaluronidases are endoglycosidases that catalyze HA depolymerization via cleavage of the β -N-acetyl-D-glucosaminidic bonds. In humans, five hyaluronidase genes and one hyaluronidase pseudogene has been described [2]. The human hyaluronidase genes are clustered on chromosome 3p21.3 (Hyal1, Hyal2 and Hyal3) and chromosome 7q31.3 (Hyal4, Hyal1 pseudogene and PH20). Thus, the hyaluronidase genes appear to have resulted from duplication. The deduced hyaluronidase amino acid sequences show that these enzymes share approximately 40% identity [3]. It is well known that a number of cancer cell types overexpress hyaluronidase enzymes including prostate [4], bladder carcinoma [5], squamous cell head and neck cancer [6] and malignant melanoma [7]. The increased activity of the hyaluronidases has been correlated with several tumor cell behaviors including tissue invasion [8], resistance to apoptosis [9] and the potentiation of angiogenesis [7].

The presence of HA-ase may be monitored by utilizing its digestion of fluorescently labeled HA. One approach is to measure the changes in polarization of fluorescence from labeled HA as it is digested by HA-ase, but this method produces changes too small for reliable detection [10]. Another approach involves dually labeling HA molecules with a pair of fluorophores suitable for Förster Resonance Energy Transfer (FRET). In this case, one fluorophore (the donor) will absorb the supplied excitation light and transmit this energy through a non-radiative process to the second fluorophore (the acceptor), which will then release the energy as light according to its characteristic emission spectrum. As long as the two molecules are close to each other, the short wavelength emission of the donor will be quenched in favor of the long wavelength emission of the acceptor. When the HA molecule is cleaved, the FRET is released and donor dominates the emission spectrum. Such ratiometric detection techniques are much more sensitive to the presence of HA-ase, but involve dual labeling of HA [11, 12].

Recently we proposed a similar FRET-based technique for the detection of HA-ase which involved only one fluorophore. It is well known that fluorescein has the ability to quench itself through a process called homo-FRET [13–15]. The homo-FRET Förster distance, at which there is a 50% probability of energy transfer, is about 42 Å for fluorescein, which is comparable to the size of many proteins [16]. Thus a single macromolecule containing more than one fluorescein molecule is expected to experience homo-FRET. This process is connected to a substantial decrease in fluorescence emission along with an increase in radiative decay (fluorescence lifetime). Thus we have presented a lifetime-based method by which the amount of homo-FRET observed is linked to the amount of HA-ase present [13]. This method has advantages over intensity based methods, namely that the partially quenched lifetime of fluorescein is an absolute quantity that does not depend on the measurement platform or excitation power. However, this method is not without drawbacks. The biggest drawback is the expense of time correlated single photon counting (TCSPC) equipment necessary for lifetime-based sensing and subsequently the lack of abundance of this equipment. Pulsed laser sources are necessary for TCSPC, and they are many-fold more expensive than most continuous wave lasers, depending on numerous factors. While it is true that FCS requires extremely small detection volumes thus adding to the cost of a dedicated FCS system, the benefit of FCS is that it may be performed on equipment already in place in many laboratories. A simple APD detector connected to an unused microscope port and software for analysis are all that is needed for FCS collection. No laser scanning systems are necessary, and the fiber connecting the detector provides a “pinhole” through which unfocused light is eliminated. Also, many new laser scanning confocal microscopes are being sold with GaAsP hybrid detectors whose sensitivities are already sufficient for FCS. So FCS analysis may be performed with equipment widespread throughout biological laboratories after only minimal upgrades.

In this report, we present a method to detect the activity of HA-ase using Fluorescence Correlation Spectroscopy (FCS) and HA heavily loaded with fluorescein (HA-FI). This technique detects changes in intensity as homo-FRET in HA-FI is released, it detects changes in the diffusion of HA-FI fragments in solution as they are cleaved into smaller pieces, and it detects changes in concentration as one HA-FI molecule is cut into many, smaller fragments. FCS is capable of detecting all of these changes simultaneously using a very small (50 μ L) sample of a very dilute (1 nM) HA-FI solution. Furthermore, it does not necessitate lifetime measurement either through time-domain or frequency-domain equipment, providing a significant cost savings.

Materials and Methods

Sodium hyaluronate from bacterial fermentation was obtained from Acros Organics (Thermo Fisher Scientific, NJ, USA). Fluorescein amine, dimethyl sulfoxide (DMSO), guanidine hydrochloride, acetaldehyde, cyclohexyl isocyanide, Sephadex G-75, and bovine testes hyaluronidase (EC 3.2.1.35, type 1-S, 451 U/mg) all were obtained from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (PBS) was purchased from Invitrogen Life Technologies (Invitrogen corporation, CA, USA) and was adjusted to pH 6.0 with 0.1 N HCl after reconstitution in deionized water (dH_2O). Slide-A-Lyser dialysis cassettes (10,000 molecular weight cutoff) were purchased from Pierce Chemical (Thermo Fisher Scientific).

Preparation of Hyaluronan-Fluorescein Probe (HA-FI)

Hyaluronan was covalently conjugated to fluorescein by methods previously described in [17]. To briefly summarize, HA was dissolved to 1.25 mg/mL in dH_2O . DMSO was added to the HA solution to create a 1:2 dilution. A pre-dissolved solution Fluorescein amine in DMSO was added to create a final concentration of 5 mg/mL. Acetaldehyde and cyclohexyl isocyanide were added to 0.04% (v/v), and the reaction was allowed to proceed for 16 h at 25 °C. Afterward, the solution was diluted 1:14 in ethanol/guanidine HCl (50 μ l of 3 M guanidine HCl per 900 μ l of 100% ethanol) and the HA was allowed to precipitate overnight at –20 °C. The precipitate was then dissolved in 1 mL of dH_2O , followed by extensive dialysis against dH_2O .

Fluorescence Correlation Spectroscopy

Fluorescence correlation spectroscopy (FCS) provides an analysis of fluorescence fluctuations over time from one or a small number of molecules in a microscopic detection volume. Usually these fluctuations are provided by fluorescent molecules diffusing through the detection volume which is placed inside of an aqueous solution. If too many molecules are located in the detection volume at any given time (i.e., the concentration is too high or the detection volume is too large), fluctuations will average out to the mean. Thus the method is highly dependent on the number of molecules observed at any one moment. The general expression for the auto- and cross- correlation is as follows [18, 19]

$$G_{ij}(\tau) = \frac{\langle \delta F_i(t) \delta F_j(t+\tau) \rangle}{\langle F_i(t) \rangle \langle F_j(t) \rangle} \quad (1)$$

where δF_i and δF_j denote the variance of the fluorescence signal about the mean at time t and a later time, $t + \tau$, for two fluorescent time traces, i and j . Also $\langle F_i \rangle$ and $\langle F_j \rangle$ are the time averaged fluorescent signals. In order to extract physical data from the correlation, a model of diffusion, G_D , is fit in terms of the lag time, τ . The simplest of these models is the expression for pure diffusion of m fluorescent species [20]

$$G_D(\tau) = G(0) \sum_{m=1}^n \left(1 + \frac{\tau}{\tau_m}\right)^{-1} \left(1 + \frac{\tau}{\tau_m} \frac{\omega^2}{z^2}\right)^{-\frac{1}{2}} \quad (2)$$

where ω is the width of the detection volume, z is the height of the detection volume, τ_m is the average diffusion time of the m^{th} diffusing species, and $G(0)$ is the correlation at $\tau = 0$, which is equal to the inverse of the average number of independent, diffusing molecules passing through the detection volume, N .

$$G(0) = \frac{1}{\langle N \rangle} \quad (3)$$

Of course, other phenomena, such as molecule orientation and antibunching, may contribute to the fluctuation of the fluorescence signal, but these phenomena occur at much shorter lag times and therefore do not affect the fitting of the diffusion model. However, the transition of an excited molecule to a triplet state will cause blinking, leading to erroneously short diffusion times and increased $G(0)$. So the following term must be added to the diffusion model to account for a single triplet (dark) state with lifetime τ_T [20, 21]

$$\left[1 - T + T e^{\left(-\frac{\tau}{\tau_T}\right)}\right] \quad (4)$$

Inserting Equation (4) into Equation (2) and simplifying the result to assume only one diffusing species with average diffusion time τ_1 yields

$$G_D(\tau) = \left[1 - T + T e^{\left(-\frac{\tau}{\tau_T}\right)}\right] G(0) \left(1 + \frac{\tau}{\tau_1}\right)^{-1} \left(1 + \frac{\tau}{\tau_1} \frac{\omega^2}{k^2}\right)^{-\frac{1}{2}} \quad (5)$$

The diffusion constant, D , may then be calculated as

$$D = \frac{\omega^2}{4\tau_1}$$

For more detailed derivations of these models, we refer the reader to [18, 20, 21].

Measurements were conducted on a Microtime 200 system from PicoQuant, GmbH (Berlin, Germany). The system was arranged as the shown in Figure 1. Excitation was provided by a 470 nm pulsed laser diode, which was directed into the sample by a 60x 1.2 NA water immersion objective, part of an Olympus IX71 microscope. Scattered light was removed by a 488 nm long pass filter, and the light passed through a 50 μm pinhole before being split by a 1:1 plate. The split light beams were then directed into two identical Single Photon Avalanche Detectors from Perkin Elmer (SPCM-AQR-14). The data from the two detectors were cross-correlated to eliminate afterpulsing [22, 23]. All data processing was performed by the SymPhoTime software, version 5.3.2, also from PicoQuant. Some of the results were supported by a complementary analysis of the same data performed using the Fluctuation Analyzer TZ software package (ISS, Champagne, IL) developed by Zeno Foldes-Papp and Tiefeng You (see supplementary data). Fluorescein is known to exhibit a blinking behavior due to population of a triplet state [24–25]. The laser power was therefore kept as low as

possible, less than $2\ \mu\text{W}$, in order to minimize this behavior [26, 27]. Equation (5) was fitted to the correlated data by the SymPhoTime software.

The HA-FI substrate was first diluted down to concentrations appropriate for FCS measurements with the addition of PBS pH=6. The concentration of HA-FI particles was later determined by FCS to be 0.9 nM. It must be noted that this measurement is independent of the number of fluorescein molecules bound to each HA molecule. The substrate was divided into 330 μL portions, each in an individual 1 mL centrifuge tube. To each of these tubes was added 20 μL of HA-ase in various concentrations. For the two hour measurements, 300 μL of this solution was dropped onto a No. 1 glass coverslip from Menzel-Gläser (Gerhard Menzel GmbH, Braunschweig, Germany) immediately after the addition of HA-ase. The large sample volume was used to reduce the effects of evaporation over two hours. Using the backscattered diffraction pattern, the focal volume was adjusted to 20 μm above the top surface of the coverslip. The 10 minute measurements differed in that each solution was incubated for 25 min after the addition of HA-ase. Then 50 μL were dropped onto the coverslip for the 10 minute measurement.

The detection volume of the system is dependent upon many factors: laser power, objective, the setting of the correction collar on the objective, the index of refraction of the measured solution, the thickness of the coverslip, etc. Precisely knowing the size of the detection volume is critical to determine the concentration and diffusion coefficient of the sample, so the detection volume was calibrated by measuring the diffusion properties of free fluorescein dye, whose diffusion coefficient has been well established [28].

Results and Discussion

Observation of HA-FI cleavage

As the HA-FI macromolecules are cleaved by HA-ase, the self-quenching due to homo-FRET is released. Thus the fluorescence intensity quickly increases after the enzyme is added to the substrate. Figure 2 shows the fluorescence time trace collected over 2 hours, where the intensity is summed in 10 minute bins. Figure 3 shows three sample FCS curves calculated from 3 points during the digestion of HA-FI. The FCS curves were calculated by performing the cross correlation, Equation 1, on the intensity time traces and fitting the diffusion model, Equation 5, to the cross correlation. Similarly, the greatest differences in the rate of diffusion happen immediately after the addition of the enzyme, as is evident by the manner in which the curves shift toward shorter lag times.

In order to accurately describe the motion of fluorescent particles diffusing through a microscopic detection volume, it is essential to study solutions with very low concentrations. When the concentration is too high, there are no fluctuations in the fluorescence signal at the microsecond range--particles enter the detection volume at the same time as particles exit the detection volume. However, low concentrations result in weak signals and/or longer collection times as well as pipetting errors from working with such dilute solutions. Fast dynamic processes are difficult to observe with long collection times, and so a balance must be struck between collection time and concentration. It was determined from preliminary experiments that substrate concentrations around 1 nM were sufficiently low to provide good correlations even after cleavage increased this concentration to around 15 nM. It must be mentioned that the concentration necessary for a good correlation depends on the detection volume of the microscope, which was 0.4 fL in our microscope. Figure 4 depicts data collected from a 1.7 nM starting solution of HA-FI as the substrate is cleaved by HA-ase over two hours. The fluorescence data was cut into sections of 2 minutes, 5 minutes, and 10 minutes, the FCS curves were fitted to each section as was done in Figure 3, and the concentration of fluorescently labeled HA-FI was calculated at each time point by Equation

3. The data was fitted to the Michaelis-Menten equation [29], and the χ^2 values improved with increasing bin width. Thus 10 minute collections were deemed appropriate for obtaining adequate statistics in FCS analyses. Furthermore, Figures 2A–4A demonstrate that the enzyme reaction occurs quickly and then levels off. Therefore we chose to collect FCS data in the relatively flat area between 25 min and 35 min after the addition of HA-ase to minimize errors incurred by the initially fast digestion of HA-Fl.

Quantification of HA-ase

In this report we have shown that the combination of FCS and heavily loaded HA-Fl provides a very sensitive means for detecting the presence of HA-ase. We now show that FCS is also able to quantitate the concentration of HA-ase present. The simplest method of quantification is to observe the correlation at $\tau = 0$, or $G(0)$, which decreases as $1/N$ and changes dramatically when N is small. While this quantity depends on the size of the observation volume, it fortunately does not require precise measurement of this volume, which is often difficult. In the FCS traces shown in Figure 5, $G(0)$ is indicated by the intercept with the vertical axis, and it is easily seen that the most substantial changes in $G(0)$ occur between the smallest increments of enzyme concentration, thus highlighting the sensitivity of the method. We conducted experiments with 12 different concentrations of HA-ase, but for clarity, only 8 are shown in Figure 5. The complete set of $G(0)$ data is listed in the second column of Table 1. Each measurement was repeated 2–4 times, and the standard deviation is listed in the third column. When $G(0)$ values are plotted versus enzyme concentration, a calibration curve is formed that allows the determination of enzyme levels in an unknown sample, see Figure 6. If precise knowledge of the detection volume is known, $G(0)$ may be used to calculate the concentration of HA-Fl, and the results are shown in columns 4 and 5 of Table 1 and graphically in Figure 7. As a secondary method, one may use the average intensity collected from the detection volume over the course of the FCS measurements (columns 8 and 9 of Table 1) to quantitate the HA-ase levels. As is shown in Figure 8, the increasing intensity corresponds to the increasing concentration of HA-Fl fragments, though greater uncertainty exists in experiments with the highest concentrations of enzyme. Analysis of the diffusion rates also holds the potential to provide a third independent measure of HA-ase concentration. However, this method is much more susceptible to errors, not only in pipetting but also in fitting the diffusion model to the correlation data, see Figure 9. Over time as the HA-Fl particles are cleaved, the detection volume is filled with particles of many different sizes for which an adequate diffusion model is difficult to construct. Furthermore, large changes in molecular weight correspond to rather small changes in the rate of diffusion [19]. So one may clearly see a difference in the diffusion rate as the HA-Fl is initially cleaved, but smaller changes due to prolonged digestion or increased levels of HA-ase result in diffusion rate differences that are overwhelmed by the uncertainties involved in fitting the diffusion model.

As a test of these methods, we collected an FCS curve from a newly prepared substrate incubated with 10 U/mL of HA-ase. The concentration of HA fragments extracted from the FCS curve was 11 nM. From the calibration curve in Fig. 7, a 10 nM concentration of the HA-FL substrate fragments predicts an 11.4 U/mL concentration of HA-ase, matching the enzyme concentration measured to within 14%. However, average intensity could not be used because of differences in the equipment setup between the measurement of data for the calibration curve and the measurement of data for this test. In order to use average intensity for quantification of HA-ase, one must form a calibration curve before each assay is performed, as is standard practice with other assays. Nonetheless, this demonstrates another benefit of our proposed FCS-based sensing, as the concentration of fluorescent substrate fragments is independent of hardware conditions, as long as the detection volume remains constant and/or is well determined.

In conclusion, FCS provides a reliable method of detecting the presence of HA-ase by the concurrent measurement of three quantities: $G(0)$ (related to substrate concentration), fluorescence intensity, and the rate of diffusion. Furthermore, we have demonstrated that FCS is capable of quantifying the levels of HA-ase detected in the solution. Especially when monitoring $G(0)$, the FCS method is very sensitive to very low concentrations of HA-ase. The FCS method of detection is also beneficial in that only minute volumes (here 50 μL) of a very dilute solution (here less than 1 nM) are needed for the substrate, thus reducing the cost for widespread testing. Also, most confocal microscopes can be easily upgraded to perform FCS with relatively low cost.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- We describe Fluorescence Correlation Spectroscopy-based sensing of hyaluronidase.
- This method allows the quantification of HA-ase through 3 independent quantities which are collected simultaneously.
- Propose that HA-FI is a useful substrate for HA-ase sensing.

Figure 1.

Fluorescence cross correlation setup. The light from a 470 nm laser diode is directed into the sample by a dichoric mirror and a 60x 1.2 NA water emersion objective. The emitted fluorescence passes through the dichroic, tube lens, pinhole, and second tube lens. The emission is then split by a 1:1 plate mirror, and each of the two beams is recorded by an avalanche photo diode. The fluorescence traces are then cross-correlated by the software.

Figure 2.

Intensity increase during cleavage. (Black) Measurement began shortly after HA-ase was added to the HA-FI substrate. Throughout the 2 hour collection, the intensity from the detection volume increased as the HA-FI was cleaved and the quenching was released. The data shown here is the combination of data collected continuously by two identical detectors arranged in the manner of Fig. 1 and then binned in increments of 10 minutes. (Red) as a control, free fluorescein dye of similar starting concentration was monitored for 2 hours.

Figure 3.

Normalized correlation functions collected from HA-FI. The data was collected in 10 minute intervals before the addition of enzyme (black), 0–10 minutes after (blue), and 110–120 minutes after the addition of 50 U/mL HA-ase (blue).

Figure 4.

Concentration of HA-FI particles in the detection volume throughout the cleavage process. The first three panels show the change in $G(0)$ throughout 2 hours of digestion with data binned in 2 minute (a), 5 minute (b), and 10 minute (c) sections. The bottom panels show the changes in concentration throughout 2 hours of digestion as calculated from the observation volume (constant) and the $G(0)$ values corresponding to each binning size: (d) 2 minute binning, (e) 5 minute binning, and (f) 10 minute binning.

Figure 5.

FCS traces from various concentrations of enzyme. Panel (a) shows the lowest four concentrations of HA-ase, and Panel (b) shows four more concentrations up to 300 U/mL. Each set of raw data is fitted to a single species diffusion model corrected for a single triplet state.

Figure 6. Dependence of $G(0)$ on the concentration of HA-ase. Each data point corresponds to a 10 minute measurement of HA-Fl after 25 minutes incubation. Error bars represent the standard deviation over 2–4 measurements for each data point.

Figure 7.

Concentration of HA-FL fragments during digestion with HA-ase. The concentrations were calculated from calibration of the detection volume with free fluorescein and the measured $G(0)$ values in Figure 6. Error bars represent the standard deviation over 2–4 measurements for each data point. As a test, 10 U/mL of HA-ase were added to a new batch of substrate. The concentration of HA-FL fragments (11 nm) predicted a 11.4 U/mL concentration of HA-ase, a 14% error.

Figure 8.

Increasing intensity with cleavage of HA. Each data point corresponds to a 10 minute measurement of HA-FI after 25 minutes incubation. The intensity over the 10 minute collection is averaged. Error bars represent the standard deviation over 2–4 measurements for each data point. The data shown here is the combination of data from two identical detectors arranged in the manner of Fig. 1.

Figure 9.

Increase in diffusion rate after cleavage of HA-FI. Each data point corresponds to a 10 minute measurement of HA-FI after 25 minutes incubation. Correlations are performed on the 10 minute measurements, and the correlations are fitted to the triplet-state corrected diffusion model. Error bars represent the standard deviation over 2–4 measurements for each data point.

Table 1

Measured and Calculated values collected by FCS. Standard deviation is calculated from 2–4 repetitions of each measurement. The HA-FI concentration values in the 4th column are complicated from the G(0) values in the second column. The diffusion constants and the average intensities were obtained independently from the FCS data

HA-se Conc. (U/mL)	G(0)	Standard Deviation (%)	HA-FI Conc. (nM)	Standard Deviation (%)	Diffusion Constant ($\mu\text{m}^2/\text{s}$)	Standard Deviation (%)	Average Intensity (cts/s)	Standard Deviation (%)
0	3.57	1	1.3	26	9	78	1135	9
1	1.98	4	2.4	16	24	24	967	21
5	0.62	48	8	49	49	19	2947	34
10	0.46	22	12	24	65	31	3870	26
20	0.32	24	14	22	76	26	5309	13
40	0.19	24	30	16	80	8	9497	7
60	0.17	12	30	17	73	8	10060	9
80	0.16	11	36	12	69	9	11576	17
100	0.16	16	36	6	72	14	10382	7
150	0.13	5	45	3	59	17	13493	22
200	0.12	23	54	17	53	38	13933	36
300	0.11	17	61	2	59	34	17889	34