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Lifetime-based sensing of the hyaluronidase using fluorescein labeled hyaluronic acid

Rafal Fudala^{1,2,‡}, Mark E. Mummert³, Zygmunt Gryczynski^{1,4}, Ryan Rich¹, Julian Borejdo¹, and Ignacy Gryczynski^{1,5,‡}

¹Department of Molecular Biology and Immunology, Center for Commercialization of Fluorescence Technologies, University of North Texas Health Science Center, Fort Worth, Texas 76107, USA. ²Department of Microbiology, Institute of Biology, The Jan Kochanowski University in Kielce, Kielce, Poland ³Department of Psychiatry and Behavioral Health, University of North Texas Health Science Center, Fort Worth, Texas 76107, USA. ⁴Department of Physics & Astronomy, Texas Christian University, Fort Worth, Texas 76129, USA ⁵Department of Cell Biology and Anatomy, University of North Texas Health Science Center, Fort Worth, Texas 76107, USA.

Abstract

In this report we propose a lifetime-based sensing (LBS) for the detection of hyaluronidase (HA-ase). First, we heavily label hyaluronan macromolecules (HA) with fluorescein amine. The fluorescein labeled HA (HA-Fl) has a weak fluorescence and short fluorescence lifetime due to an efficient self-quenching. Upon the addition of HA-ase, the brightness and lifetime of the sample increase. The cleavage of an HA macromolecule reduces the energy migration between fluorescein molecules and the degree of the self-quenching. A first order of the cleavage reaction depends on the amount of the HA-ase enzyme. We describe an HA-ase sensing strategy based on the lifetime changes of the fluorescein labeled HA in the presence of HA-ase. We demonstrate that the calibration of the sensing response is the same for the average lifetime as for a single exponential decay approximation, which significantly simplifies the analysis of the sensing measurements.

Keywords

Hyaluronan; Hyaluronidase; Homo-FRET; Fluorescence; Lifetime based sensing

Introduction

Hyaluronidases (HA-ase) are enzymes that cleave the polysaccharide, hyaluronic (HA), which is a glycosaminoglycan expressed in extracellular and pericellular matrices. In humans, five hyaluronidase genes and one hyaluronidase pseudogene has been described [1]. The hyaluronidases are endoglycosidases that predominantly catalyze hyaluronan

[‡]Address correspondence to these authors: Center of Commercialization of Fluorescence Technologies (CCFT), Dept. of Molecular Biology & Immunology, UNTHSC, Fort Worth, TX 76107, USA, and Dept. of Cell Biology and Anatomy, Fort Worth, TX 76107, USA; Tel: 8177352118; Fax: 8177352163; Rafal.Fudala@unthsc.edu; Ignacy.Gryczynski@unthsc.edu .

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depolymerization *via* cleavage of the β -N-acetyl-D-glucosaminidic bonds. In mammalian normal tissue, they are present in low concentrations; 60ng/ml in human serum [2]. It is well established that an over expression of the hyaluronidase enzymes is observed in many different cancers including prostate cancer and malignant melanomas [3, 4]. The increased activity of the hyaluronidases has been correlated with several carcinogenic cell behaviors including tissue invasion [5], resistance to apoptosis [6] and the potentiation of angiogenesis [4]. However HA-ases are also used as anticancer chemotherapeutic agents—the addition of HA-ase reduces a tumor's resistance to chemotherapy [7, 8]. HA-se can have different biological activities depending on the cancer cell type. In contrast to prostate cancer and malignant melanoma, HA-se suppresses tumorigenicity in a model of colon cancer [9].

HA, the substrate for HA-se, is a high molecular weight, linear, non-sulfated glycosaminoglycan composed of multiple subunits of D-glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) and has the primary structure $[\beta 1 \rightarrow 4 \text{GlcA } \beta 1 \rightarrow 3 \text{GlcNAc}]_n$. HA is known to exhibit diverse biological functions including: a) maintenance of tissue structural integrity, b) formation of highly hydrated matrices around individual cells, c) promotion of cellular migration including metastasis, and d) mediation of intercellular signaling. HA contributes to tumor cell behavior by: a) modulating the biomechanical properties of extracellular and pericellular matrices in which cells reside, b) forming a repetitive template for interactions with other macromolecules in the pericellular and extracellular environment and thus, contributing to the assembly, structural integrity and physiological properties of these matrices, and c) interacting with cell surface receptors through concomitant signal transduction. The digestion of fluorescently labeled HA (by HA-ase) can be used for detection of HA-ase enzyme activity. Several methods have been proposed. A simple assay for HA-ase activity using fluorescence polarization has been proposed by Murai and Kawashima [10]. However, the observed changes in polarization do not exceed 0.01 (10mP). Although polarization measurements are very precise, these changes are too small for reliable detection. Another approach involves dually labeled HA with fluorophores suitable for Forster resonance energy transfer (FRET). The cleavage of HA results in the release of FRET and change of the relative intensities of the fluorophores involved. These ratio-metric measurements offer larger signal responses in the presence of HA-ase than polarization changes but involve the dual HA labeling [11, 12].

In this manuscript we propose a simpler approach for the detection of HA-ase activity. We observed that fluorescein-labeled HA (HA-Fl) shows a very short fluorescence lifetime due to the self-quenching of fluorescein, a phenomenon known for many years. Self-quenching of fluorescein and other xantene-type dyes is one of the oldest observations in fluorescence spectroscopy and is due to resonance energy transfer between fluorescein molecules (homo FRET). This process was frequently connected to decreases in the quantum yield, lifetime and polarization of viscous solutions with high probe concentrations [13-18]. In the case of fluorescein, the Förster distance (50% probability of excitation energy transfer) for homo FRET is about 42Å [19]. Since this distance is comparable to or larger than the size of many proteins, FRET is expected to occur when a macromolecule contains more than a single fluorophore.

The digestion of HA-Fl by HA-ase enzyme releases fluorescein self-quenching, thereby increasing fluorescence brightness and lifetime. We describe here the strategy for the HA-ase detection using changes in observed lifetimes (lifetime-based sensing, LBS).

There are many advantages of LBS over intensity-based sensing methods [20, 21]. Fluorescence lifetime measurements yield absolute quantity values that are independent of the measurement platform. LBS does not depend on the excitation intensity and optical misalignments which simplifies the calibration of the sensing device. Mentioned above, LBS

properties and its robustness make this approach an ideal tool for measurements of difficult-to-control “real world” samples such as physiological fluids or tissue.

Material 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 distilled water (dH₂O). Slide-A-Lyser dialysis cassettes (10,000 molecular weight cutoff) were purchased from Pierce Chemical (Thermo Fisher Scientific).

Preparation of conjugated Hyaluronan (HA-FI Probe)

Hyaluronan was covalently conjugated to fluorescein amine essentially as described [22]. Briefly, HA was dissolved to 1.25 mg/ml in dH₂O. The HA solution was diluted 1:2 in DMSO, and fluorescein amine (predissolved as a DMSO stock solution) was added to 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 µl of 3 M guanidine HCl per 900 µl of 100% ethanol) and the HA was allowed to precipitate overnight at –20 C. The precipitate was then dissolved in 1 ml of dH₂O, followed by extensive dialysis against dH₂O.

Fluorescence Measurements of Hyaluronan Hydrolysis

The HA-FI probe (labeled hyaluronan) was incubated with a different concentration of hyaluronidase in PBS pH 6.0 at room temperature (RT). At selected time points, fluorescence emission spectra were collected using Cary Eclipse spectrofluorometer (Varian Inc., Australia). Measurements were performed in 0.4×0.4 cm quartz cells with the excitation at 480 nm and emission at 520 nm.

Lifetime Measurements of the HA-FI Probe

Fluorescence lifetime measurements were done using a FluoTime 200 fluorometer (PicoQuant, GmbH, Berlin, Germany). This time-resolved instrument is equipped with an ultrafast detector, a Hamamatsu R3809U-50 microchannel plate photomultiplier (MCP). For the excitation we used a 470 nm picosecond pulsed laser diode. The detection was made through a monochromator supported by a 495 nm long wave pass filter in order to eliminate a scattered excitation light. The decay data were analyzed with FluoFit, version 5.0 software (PicoQuant, GmbH). Fluorescence intensity decays were analyzed by reconvolution with the instrument response function and analyzed as a sum of experimental terms:

The intensity decays were analyzed with a multi-exponential model using FluoFit v. 5.0 software (PicoQuant, GmbH). The data for each experiment were fitted with the multi-exponential model:

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (1)$$

where τ_i are the decay times and α_i are the pre-exponential factors (amplitudes) of the individual components ($\sum \alpha_i = 1$). The contribution of each component to the steady state intensity is given by:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \quad (2)$$

where the sum in the denominator is over all the decay times and amplitudes. The mean decay time (intensity-weighted average lifetime) is given by:

$$\bar{\tau} = \sum_i f_i \tau_i \quad (3)$$

and the amplitude-weighted lifetime is given by:

$$\langle \tau \rangle = \sum_i \alpha_i \tau_i \quad (4)$$

Results and discussion

Recovery of the brightness upon a release of the self-quenching

Absorption and fluorescence spectra of fluorescein-labeled HA, HA-Fl, are shown in Figure 1. A convenient blue excitation results in a green emission of the fluorescein, which can be easily filtered from the excitation light. A large spectral overlap (Figure 1, shadowed area) enables an efficient homo-transfer of the excitation energy.

Heavily labeled HA-Fl shows a relatively weak green fluorescence (Figure 2, top). Upon the addition of HA-ase, the HA macromolecule is cleaved into smaller pieces. The homo-transfer of the excitation energy and the subsequent self-quenching of fluorescein moiety are reduced which results in a stronger green fluorescence (Figure 2, bottom). The change in the fluorescence spectrum in the presence of HA-ase is shown in Figure 3. At the level of 100 U/ml HA-ase, after 30 min of incubation, the brightness of the HA-Fl solution (10 $\mu\text{g/ml}$) increases about 2.5 fold. It should be noted that, in the presence of the analyte, the observed signal increases, which is a favorable future in the sensing study.

Changes to the HA-Fl fluorescence lifetimes in the presence of HA-ase

In the absence of HA-ase, the lifetime of the HA-Fl solution has a very short lifetime and the fluorescence intensity decay is very heterogeneous (Figure 4, upper right). In the presence of HA-ase, the lifetimes are longer and intensity decays less heterogeneous (Figure 4). Upon the addition of 100 U/ml of HA-ase and 30 min incubation, the amplitude averaged lifetime increases more than two-fold, similar to the increase in the brightness.

We selected the 30 min incubation time after studying the time dependent traces with different amounts of HA-ase enzyme (Figure 5). As seen from this Figure, the first order of the kinetics depends strongly on the amount of the HA-ase, and the kinetics stabilize after about 30 min. The different signal responses to the different amounts of the enzyme facilitate the HA-ase detection. The lifetime changes in the presence of HA-ase are summarized in the Table 1.

Lifetime-based sensing of HA-ase activity

In the case of complex intensity decays, it is not always obvious which lifetime parameters should be used to describe a desired dependence. In order to have changes in the lifetime consistent with the changes in the brightness, one should use the amplitude averaged lifetimes, $\langle \tau \rangle$, [25].

Using these (amplitude averaged lifetimes) from Table 1, we constructed a calibration curve for the sensing of HA-ase (Figure 6, left). Assuming the uncertainty of the measured lifetime is 0.02ns, the HA-ase activity can be estimated within an accuracy of 10%.

Although calculations of the average lifetimes from multi-exponential decay fittings pose no problem with today's computers, we were pleasantly surprised that the HA-ase dependence of lifetimes estimated from single exponential fits is almost identical (Figure 6, right). This could be a significant simplification for developers constructing a sensing device.

Since there are many researchers in the fluorescence field using frequency-domain instrumentation, we reconstructed time-domain lifetimes into the frequency-domain (Figure 7). At the frequency of 100 MHz the phase decreases almost 15 degrees, and the modulation is about 0.16 upon addition of 100 U/ml HA-ase. The phase and modulation data can be as well used for an efficient sensing of HA-ase.

In conclusion: we believe that the lifetime-based sensing of HA-ase presented above carries many advantages over intensity-based methods. The independence from excitation power and optical configuration are probably the most important considerations for the construction of a reliable detection device. Also, the range of observed lifetime changes is much more comfortable than the range of the polarization changes. The lifetime-based sensing of HA-ase can be done in the solution with the calibrated HA-Fl, using a titration method. However, for the surface measurements (skin cancers detection) one can prepare a water-based gel or cream containing HA-Fl and use a portable fluorometer for the detection.

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Highlights

- We describe Lifetime-based sensing of hyaluronidase.
- Spectroscopic evaluation of lifetime-based-sensing using steady state/time resolved fluorescence.
- Propose that HA-FI is a useful substrate for HA-ase sensing.

Figure 1.

Absorption and emission spectra of HA-Fl. The large spectral overlap (shadowed area) is responsible for an efficient excitation energy migration (homo-transfer) between fluorescein molecules. The energy migration enables the self-quenching process.

Figure 2. Schematic of the HA-FI digestion. The HA-ase enzyme cleaves HA macromolecule reducing the self quenching (SQ) of the fluorescein labels. In the effect, the brightness and lifetime of the studied sample increased.

Figure 3.

The change in the fluorescence spectrum of HA-FI after 30 min of incubation with HA-ase (100 U/ml) at the room temperature. The concentration of HA-FI was 10 $\mu\text{g/ml}$ and the excitation was 470 nm.

Figure 4.

Fluorescence intensity decays of HA-FI incubated with HA-ase for 30 min at room temperature. The concentrations of HA-FI was 10 $\mu\text{g/ml}$ in all samples, and the amounts of added HA-ase were: A – 0 U/ml, B – 1 U/ml, C – 10 U/ml, D – 100 U/ml. The average lifetime of HA-FI progressively increases and the heterogeneity of decay decreases. The broken straight lines show the best single exponential fits.

Figure 5.

The time-dependent changes in the lifetime of HA-Fl in absence and presence of HA-ase enzyme. The differences in the first order kinetics facilitate the HA-ase detection. For the sensing range 0-100 U/ml we selected a 30 min incubation time.

Figure 6.

A: Calibration curve for the lifetime-based HA-ase sensing constructed for 30 min incubation time. For a conservatively estimated uncertainty of the average lifetime, 0.02 ns, the accuracy of HA-ase detection will be about 1 U/ml in the range 0-30 U/ml. B:

Calibration curve constructed with lifetimes estimated from single exponential fits.

Figure 7.

Frequency-domain representations of the decays shown in Figure 4. At the frequency of 100 MHz the maximum changes in phase and modulation are 20 degrees and 0.2, respectively.

Table 1

Multi-exponential analysis of the HA-FI fluorescence intensity decays in the absence and presence of the HA-ase.

HA-ase	a_1	a_2	a_3	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	$\langle \tau \rangle$ (ns)	$\bar{\tau}$ (ns)	χ^2_r
0 U/ml	1	—	—	1.22	—	—	1.22	1.22	42.7
	0.640	0.270	0.009	0.12	0.75	2.41	0.49	1.38	1.0
1 U/ml	1	—	—	1.70	—	—	1.70	1.70	33.6
	0.557	0.309	0.134	0.15	0.69	2.86	0.76	1.82	1.0
10 U/ml	1	—	—	2.02	—	—	2.02	2.02	34.6
	0.533	0.308	0.159	0.16	1.07	3.17	0.81	2.13	1.0
30 U/ml	1	—	—	2.20	—	—	2.20	2.20	33.0
	0.516	0.308	0.176	0.17	1.08	3.31	1.00	2.30	1.0
50 U/ml	1	—	—	2.32	—	—	2.32	2.32	31.7
	0.510	0.304	0.186	0.17	1.13	3.41	1.06	2.41	1.0
70 U/ml	1	—	—	2.39	—	—	2.39	2.39	30.6
	0.508	0.297	0.195	0.17	1.14	3.46	1.10	2.48	1.0
100 U/ml	1	—	—	2.45	—	—	2.45	2.45	30.6
	0.506	0.293	0.201	0.17	1.14	3.48	1.12	2.52	1.0

$\langle \tau \rangle$ = amplitude-weighted lifetime (see equation 4), $\bar{\tau}$ = intensity-weighted average lifetime (see equation 3), χ^2_r = Reduced Chi-square, goodness of fit.