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ORIGINAL PAPER

Biophysical Properties of Phenyl Succinic Acid Derivatised Hyaluronic Acid

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Abstract Modification of hyaluronic acid (HA) with aryl succinic anhydrides results in new biomedical properties of HA as compared to non-modified HA, such as more efficient skin penetration, stronger binding to the skin, and the ability to blend with hydrophobic materials. In the present study, hyaluronic acid has been derivatised with the anhydride form of phenyl succinic acid (PheSA). The fluorescence of PheSA was efficiently quenched by the HA matrix. HA also acted as a singlet oxygen scavenger. Fluorescence lifetime(s) of PheSA in solution and when attached to the HA matrix has been monitored with ps resolved streak camera technology. Structural and fluorescence properties changes induced on HA-PheSA due to the presence of singlet oxygen were monitored using static light scattering (SLS), steady state fluorescence and ps time resolved fluorescence studies. SLS studies provided insight into the depolymerisation kinetics of PheSA derivatised HA matrix in the presence of singlet oxygen. Time resolved fluorescence studies grave insight into the dynamics of the reaction mechanisms induced on HA-PheSA by singlet oxygen. These studies provided insight into the medical relevance of PheSA derivatised HA: its capacity of scavenging singlet oxygen and of quenching PheSA fluorescence. These studies revealed that HA-PheSA is a strong quencher of electronic excited state PheSA and acts as a scavenger of singlet oxygen, thus medical applications of this derivatised form of HA may protect tissues and organs, such as skin, against reactive oxygen species damage.

Keywords Fluorescence lifetime · Hyaluronic acid · Hyaluronan · Fluorescence quenching · Singlet oxygen scavenger

Abbreviations

HA	hyaluronic acid
PheSA	anhydride form of phenyl succinic acid
ASA	aryl/alkyl succinic anhydrides
ASA-HA derivatives	aryl/alkyl succinic anhydrides derivatives of hyaluronic acid
HA-Phe SA 19% derivatised	hyaluronic acid derivatised with phenyl succinic acid anhydride (19% substituted)
19% labelled HA	hyaluronic acid derivatised with phenyl succinic acid anhydride (19% substituted)
SLS	static light scattering
ROS	reactive oxygen species
TMPyP photosensitizer, 5,10,15,20	Tetrakis(N-methyl-4-pyridyl)-21H, 23H-porphine as the tetra-p-tosylate salt

Introduction

Hyaluronic acid (HA), also called hyaluronan and hyaluronate, is a glycosaminoglycan distributed widely through-

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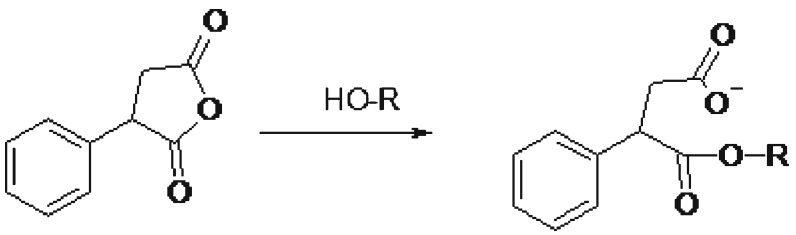
out connective, epithelial, and neural tissues. It is one of the main components of the extracellular matrix. HA helps keeping the cartilage that cushions joints strong and flexible and acts as a joint lubricator. Hyaluronan is naturally found in many tissues of the body, such as skin, cartilage, and the vitreous humour. It occurs throughout the body at locations where patients with hereditary connective tissue disorders have problems such as joints, heart valves and eyes. It is therefore well suited for biomedical applications targeting these tissues [1–7]. Hyaluronic acid is an important new compound on the biopharmaceutical market. HA has recently been studied for use in drug delivery applications [8]. HA has also been used as a surgical aid in ophthalmology and it also has therapeutic potential for the treatment of arthritis and wound healing [9]. Its beneficial properties are intimately coupled with its molecular structure and dynamics. These features are directly influenced by the length of the biopolymer chain [10, 11]. Modification of hyaluronic acid (HA) with aryl/alkyl succinic anhydrides (ASA) to produce aryl/alkyl succinic anhydride HA derivatives has been carried out particularly by cosmetic and biomedical industries. There is a need, particularly in the cosmetics and biomedical industries, for hyaluronic acid based compounds or derivatives that have certain altered characteristics as compared to non-modified HA. Properties of interest are the improved ability to stabilize foam, and the ability to blend with non-hydrophilic materials, such as is used typically in cosmetics products. The ASA-HA derivatives are expected to have interesting properties that can be used for advanced formulation (bind stronger to the skin compared to non-modified HA so that they are not so easily washed of), possibly also in drug delivery systems by encapsulation (nano/micro capsules) or formation of nano/micro spheres [12]. Further, the low MW ASA-HA derivatives are expected to penetrate the skin more efficiently than non-modified HA of the same MW [12].

Certain environmental factors may change the intrinsic length of the biopolymer. Among these factors one stands out as being highly significant: free radicals, specifically reactive oxygen species (ROS). Species formed due to oxygen reduction, O^{2-}, OH radicals and H_2O_2, together with singlet oxygen $^1O_2$ are referred to as ROS. Because of their high reactivity these compounds are relatively short-lived. The lifetime of singlet oxygen in tissues is reported to be ~3–4 μs, which allows for diffusion of hundreds of nm in cells and tissues [13–15]. Many diseases are linked to damage from ROS as a result of an imbalance between radical-generating and radical-scavenging systems—a condition called oxidative stress. Oxidative damage to biological systems, either accidental or intended, is a major cause of cell death and aging [16]. When exposed to reactive oxygen species hyaluronic acid may de-polymerize, and as a consequence some of its beneficial properties may change [17–21]. In some circumstances, this in itself may be advantageous and used for the protection of other tissue components since hyaluronic acid will act as a radical scavenger. Hyaluronic acid (HA) has been shown to protect tissues from oxygen free radical damage. The mechanism behind this protection seems to involve free radical depolymerization of hyaluronic acid [22, 23].

In the present study, HA has been derivatised with phenyl succinic acid according to the scheme below, where R can be any primary or secondary hydroxyl group on the two repeating monosaccharides in the HA structure:



The fluorescence lifetime of the PheSA in solution and when attached to the HA matrix has been monitored with ps resolved streak camera technology. Our studies reveal that hyaluronic acid is a quencher of PheSA. Furthermore, singlet oxygen induced depolymerisation of derivatised HA by has also been has been monitored with static light scattering. Singlet oxygen was generated upon 420 nm illumination of the sensitizer TMPyP. HA depolymerisation. Our studies reveal that derivatised hyaluronic acid is a scavenger of singlet oxygen. Both fluorescence quenching and singlet oxygen scavenging properties are medically relevant and therefore of interest to pharmaceutical companies.

Materials and methods

Static light scattering and steady state fluorescence studies

Samples

PheSA (Phenyl succinic acid anhydride), HA-Phe SA 19% substituted (hyaluronic acid derivatised with phenyl succinic acid anhydride, Novozymes batch EXT-2006-00047-3), TMPyP (photosensitizer, 5,10,15,20-Tetrakis(N-methyl-4-pyridyl)-21H, 23H-porphine as the tetra-p-tosylate salt).

Static light scattering (SLS)

HA has been dissolved in a 100 mM phosphate buffer at pH 7.5. HA was allowed to stabilise after solubilisation for at least 2 hours. HA stock solution was prepared at a concentration of 10 mg/ml. Solubilisation was done at room temperature on a shaking platform at 150 rpm. Prior to measurements 1 mL of the HA solution was diluted with 2 mL phosphate buffer.

Formation of reactive singlet oxygen species

Singlet oxygen was generated upon 420 nm illumination of the sensitizer TMPyP. The samples consisted of: 200 μl HA 19% substituted (10 mg/ml) in 100 mM phosphate buffer (pH 7.5) and 4 μl or 8 μl TMPyP (2.8 mg/ml) in 100 mM phosphate buffer, pH 7.5.

Static light scattering was used to quantify HA depolymerization in the presence of singlet oxygen. Possible degradation of HA by reactive oxygen species leads to fragmented HA that scatters less the incoming light. Measurements were carried on in a Photon Technology International fluorescence spectrometer. Four hundred twenty nanometer incident light (4 nm band pass slit) was selected from a 75 W Xenon lamp by means of a monochromator and sent through a neutral density optical filter into the sample. Line voltage was controlled and maintained at 4 V, thus avoiding fluctuations deriving from the power coming from the electrical outlet. The light scattered from the sample was collected at 90° angle from the excitation light. The geometry of the system is the same as when carrying on steady state fluorescence measurements. The sample was stirred at 400 rpm. The intensity of scattered light by HA in solution was monitored in the absence and in the presence of singlet oxygen. See further details under results section.

Steady state fluorescence

Fluorescence measurements of hyaluronic acid derivatised with phenyl succinic acid anhydride (HA-PheSA 19% derivatised) were carried on in a Photon Technology International fluorescence spectrometer. Derivatised HA has been dissolved in 100 mM phosphate buffer at pH 7.5 to a final concentration of 10 mg/ml. Prior to measurements 1 mL of the HA solution was diluted with 2 mL phosphate buffer. Incident light (5 nm band pass) was selected from a 75 W Xenon lamp by means of a monochromator. Line voltage was controlled and maintained at 4 V, thus avoiding fluctuations deriving from the power coming from the electrical outlet. The emitted fluorescence light from the sample was collected in a 90° angle from the excitation light. The HA-PheSA samples were stirred at 700 rpm. All slits were kept at 5 nm. Fluorescence emission spectra were acquired upon 280 nm excitation. Fluorescence excitation spectra were acquired keeping emission wavelength at 350 nm.

Streak camera studies

Samples

19% derivatised HA-PheSA in the absence of sensitizer: 10 mg/ml in 100 mM phosphate buffer, pH 7.5. PheSA in the absence of sensitizer: 1 mg/ml in 100 mM phosphate buffer, pH 7.5. Experiments using 19% derivatised HA-PheSA in the presence of sensitizer: 200 μl HA 19% (10 mg/ml) in 100 mM phosphate buffer, pH 7.5, 1,3 μl TMPyP (from 2.8 mg/ml stock) in 100 mM phosphate buffer, pH 7.5.

Illumination protocol

Streak camera fluorescence spectroscopy of 19% derivatised HA and PheSA after exposure to singlet oxygen

Time-resolved measurements were carried on after excitation by ultra-short laser pulses at 280 nm. The pulses were generated by sending the output of a Spectra-Physics Tsunami 3960 laser (<100 fs pulse duration, 12 nm FWHM, 80 MHz repetition rate, λ=840 nm pumped by a 5 W solid state laser Millennia V at 532 nm from Spectra Physics) through a pulse picker, which decreased the pulse repetition rate to 8 MHz. The fundamental pulse was mixed with its second harmonic (420 nm) in a frequency doubler/tripler (GWU; Spectra Physics) to generate 280 nm light pulses. The power at 280 nm was ~1.1 mW. Fluorescence emission was collected perpendicularly to the excitation beam through an input slit (100 μm) of a spectrograph (Oriel, MS257) with a grating blazed at 300 nm with 300 lines/mm after which it was focused into the slit of the input optics (100 μm slit) of the streak camera (Optronis, GmbH). Data were acquired for 1.8 ns with 2.6 ps resolution.

Streak camera fluorescence spectroscopy of 19% labelled HA and PheSA after exposure to singlet oxygen

Prior to acquiring the time-resolved fluorescence streak data of labelled HA and PheSA, the sample was pre-irradiated with 420 nm light. The purpose was to excite the sensitizer TMPyP present in solution in order to generate singlet oxygen, a reactive oxygen species. The pulses were generated by sending the output of a Spectra-Physics Tsunami laser as described above, through a pulse picker, which decreased the pulse repetition rate to 8 MHz. The second harmonic (420 nm) of the fundamental was generated in a frequency doubler (GWU; Spectra Physics).

The power at 420 nm was ~1.7 mW. After singlet oxygen generation, time-resolved measurements were carried on upon excitation of PheSA and labelled HA by ultra-short laser pulses at 280 nm. Fluorescence emission was collected perpendicularly to the excitation beam through an input slit (100 μm) of a spectrograph (Oriel, MS257) with a grating blazed at 300 nm with 300 lines/mm after which it was focused into the slit of the input optics (100 μm slit) of the streak camera (Optronis, GmbH). Data were acquired for 1.8 ns with 2.6 ps resolution.

Data analysis

Scattered light intensity decays and fluorescence intensity decays were fitted to an exponential model (Eq. 1) in Origin 8. The goodness of the fit, evaluated by the χ^2-value, the errors associated to each fitted parameter and the residual plots, was used to decide, whether a single or a double exponential model was appropriate.

$$I(t) = \sum_i \alpha_i \cdot \exp\left(\frac{-t}{\tau_i}\right) + I_0 \quad (1)$$

where I(t) is the intensity decay, α_i is the amplitude (pre-exponential factor), τ_i the decay lifetime of the i-th discrete component and $\sum\alpha_i=1.0$. Fluorescence mean lifetimes have been calculated as previously described [24].

Results

In Fig. 1a are displayed the excitation spectra of both PheSA and HA-PheSA, with emission set at 350 nm. It can be observed that the fluorescence excitation of phenyl succinic acid anhydride (PheSA) is ~10 nm blue shifted compared to the fluorescence excitation of HA-PheSA. In Fig. 1b are displayed the fluorescence emission spectra of both PheSA and HA-PheSA, upon excitation at 280 nm. It can be observed that HA-PheSA displays a peak centered at 351 nm, while PheSA spectra has two distinct peaks, one centered at 356 nm and another broad peak at ~430 nm. The HA-PheSA 351 nm peak is ~6 nm blue shifted compared to the peak 356 nm peak observed in PheSA.

In Fig. 2a are displayed the time and frequency resolved fluorescence emission streak camera spectra of PheSA and HA-PheSA as 2D graphs. Fluorescence has been collected during the initial 1.8 ns after the excitation pulse. Fluorescence emission intensity is color coded from white to black (red to dark blue in color figures), indicating white color (red in color figures) high fluorescence intensity and black color (dark blue in color figures) low fluorescence intensity. It can be observed that the PheSA sample has a longer average fluorescence lifetime (89 ps) compared to

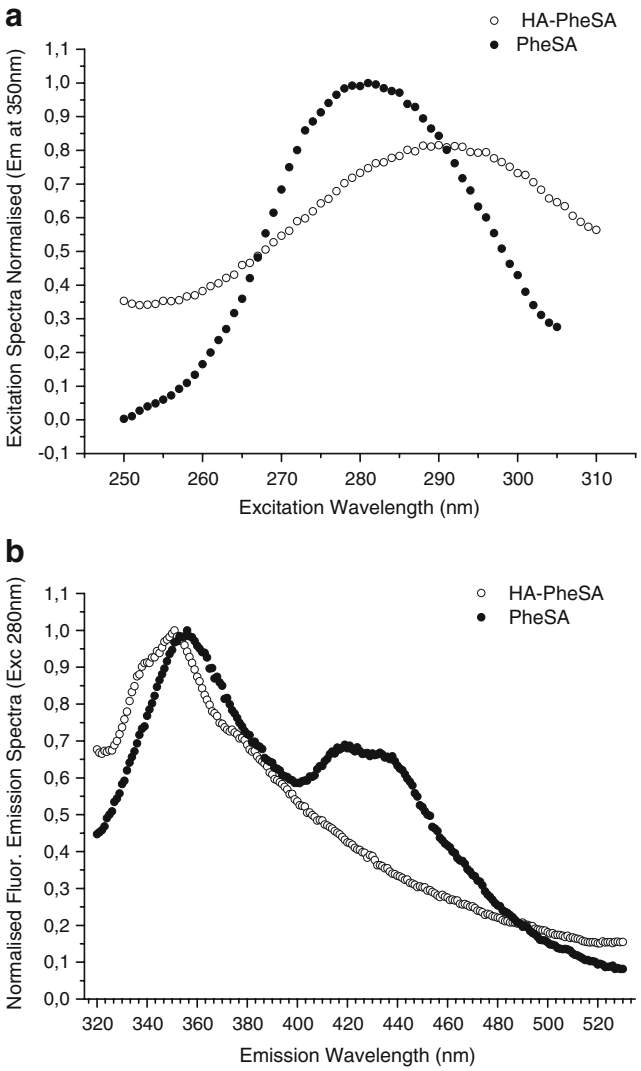


Fig. 1 Fluorescence excitation **a** and emission **b** spectra of PheSA (Phenyl succinic acid anhydride) when part of the hyaluronic acid matrix (HA-PheSA 19% derivatised, open circles) and when alone in solution (black solid circles). Excitation spectra were acquire fixing the emission wavelength at 350 nm. Emission spectra were acquired exciting the sample with 280 nm light. Samples were prepared in 100 mM phosphate buffer at pH 7.5. See methods section for details

the average HA-PheSA fluorescence lifetime (20 ps) (see Table 1). The fluorescence lifetimes and pre-exponential factors recovered from fitting the fluorescence decay at the wavelength of maximum fluorescence intensity for PheSA and HA-PheSA sample are displayed in Fig. 2b, c, respectively and in Table 1. PheSA fluorescence data was best fit with a single exponential model and the recovered fluorescence lifetime was ~89 ps, while HA-PheSA was best fit by a double exponential decay model with recovered average lifetime of 20 ps. A single exponential fit of the HA-PheSA fluorescence decay could not fit the data: R^2 value would decrease to 0,91 compared to 0,99 (two exponetial fit) and residuals become larger and highly

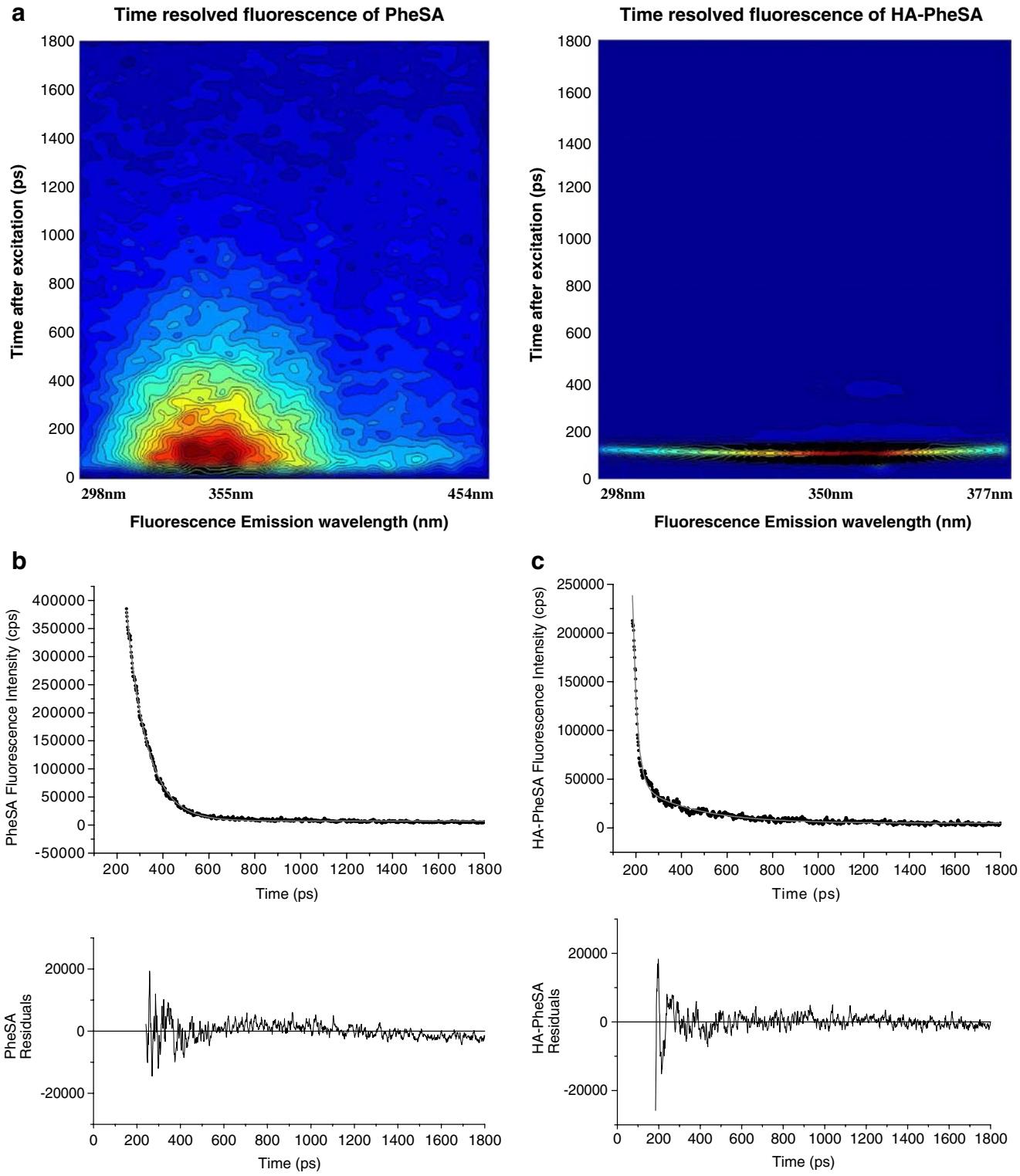


Fig. 2 **a** Two-dimensional representation of a temporally and spectrally resolved streak camera image of the fluorescence emission decay of: left) PheSA (Phenyl succinic acid anhydride) upon 280 nm excitation; right) hyaluronic acid matrix labeled with PheSA upon 280 nm excitation; **b** Single exponential decay fitting of the fluorescence decay trace of PheSA at the wavelength of max fluorescence intensity. Data points are represented as solid circles and the fitted data as a grey line. The recovered lifetime is ~89 ps; **c** Bi-exponential decay fitting of the fluorescence decay trace of HA-PheSA at the wavelength of max fluorescence intensity. Data points are represented as solid circles and the fitted data as a grey line. The dominant fluorescence lifetime recovered is ~20 ps. See Table 1

Table 1 Fluorescence lifetimes and pre-exponential factors recovered from fitting the fluorescence decay at the wavelength of maximum fluorescence intensity for PheSA and HA-PheSA samples (Fig. 2a, b and c) , and HA-PheSA after exposure to singlet oxygen (Fig. 3a, b) (see "Materials and Methods" for details)

Sample	Lifetimes and pre-exponential factors		Reduced χ^2	R^2
	τ_1 [ps]	τ_2 [ps]	1,9e8	0,982
PheSA	89±0	–		
	α_1	α_2		
	1.0	–		
	τ_1 [ps]	τ_2 [ps]		
HA-PheSA	19.8±0.3	248±6	7,4e6	0,985
	α_1	α_2		
	0,999±0,012	0,001±0,0002		
	τ_1 [ps]	τ_2 [ps]		
HA-PheSA after exposure to singlet oxygen	37±1	952±64	7,8e8	0,997
	α_1	α_2		
	0,98±0,02	0,02±0,001		

trendy (data not shown). In Fig. 3a is displayed the time and frequency resolved fluorescence emission streak camera spectra of HA-PheSA after exposure to singlet oxygen. It can be clearly seen that the presence of singlet oxygen has led to the appearance of a long fluorescence lifetime component not present in the absence of singlet oxygen. The fluorescence lifetimes and pre-exponential factors recovered from fitting the fluorescence decay at the wavelength of maximum fluorescence intensity for HA-PheSA sample after exposure to singlet oxygen shown in Fig. 3b is displayed in Table 1. The fit of the fluorescence decay at the wavelength of maximum fluorescence intensity for HA-PheSA after exposure to singlet oxygen is displayed in Fig. 3b. The data was best fit with a double exponential decay model and the recovered fluorescence lifetimes were 37 ps (98% of the population) and 952 ps (2% of the population). The average lifetime was 266 ps. A double exponential fit of the data while fixing one or both lifetimes and making them identical to the recovered lifetimes observed for HA-PheSA lead to a very poor fit, with trendy large residuals, large R^2 and large errors (data not shown).

In Fig. 4a is displayed the static light scattering intensity data set obtained upon irradiating the HA sample with 420 nm light prior and after the addition of TMPyP, the photosensitizer that upon 420 nm will lead to the formation of singlet oxygen. At the selected wavelength (420 nm) we

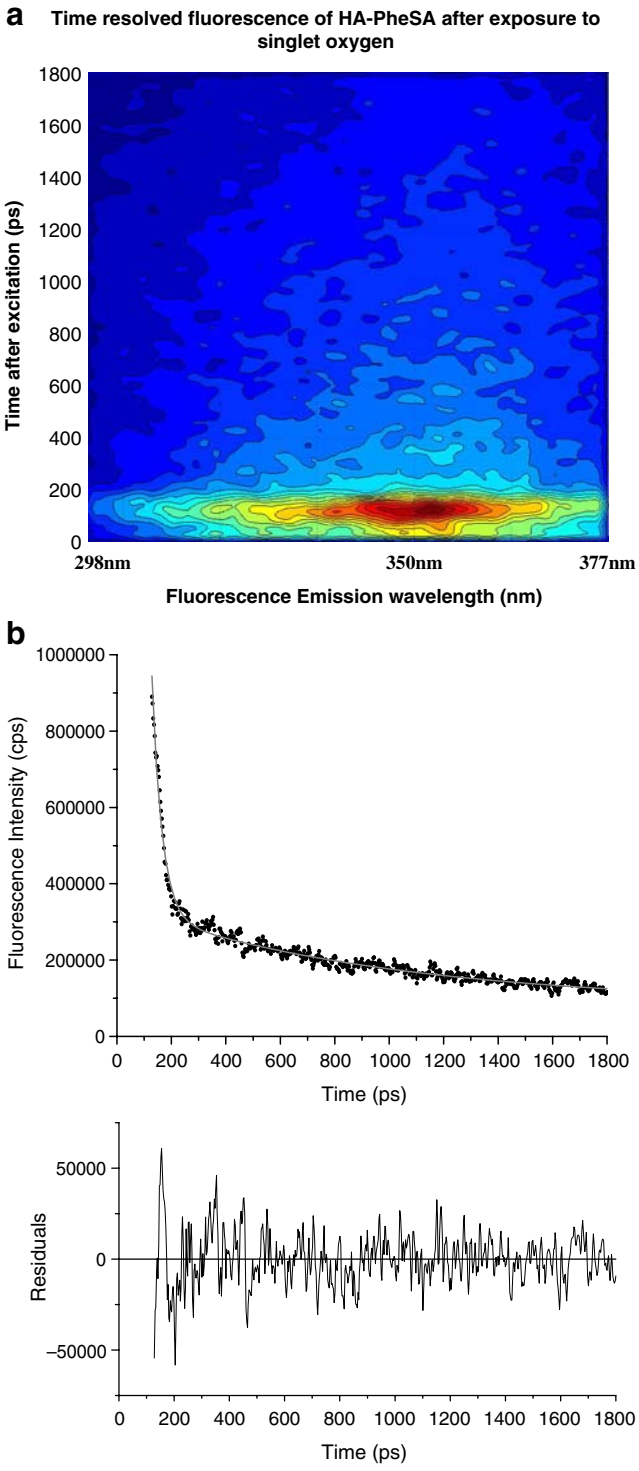


Fig. 3 **a** Two-dimensional representation of a temporally and spectrally resolved streak camera image of the fluorescence emission decay of hyaluronic acid matrix labeled with PheSA (Phenyl succinic acid anhydride) upon 280 nm excitation, after exposure to singlet oxygen. Singlet oxygen has been generated prior to data acquisition, upon 420 nm illumination of the photosensitizer TMPyP during 5 min prior to data acquisition. When comparing this data with the data displayed in Fig. 2a, right panel, it can be seen that the presence of singlet oxygen lead to a longer fluorescence lifetime of HA-PheSA; **b** Bi-exponential decay fitting of the fluorescence decay trace of HA-PheSA after TMPyP excitation for 5 min,. Data points are represented as solid circles and the fitted data as a grey line. The dominant fluorescence lifetime recovered is ~37 ps. See Table 1

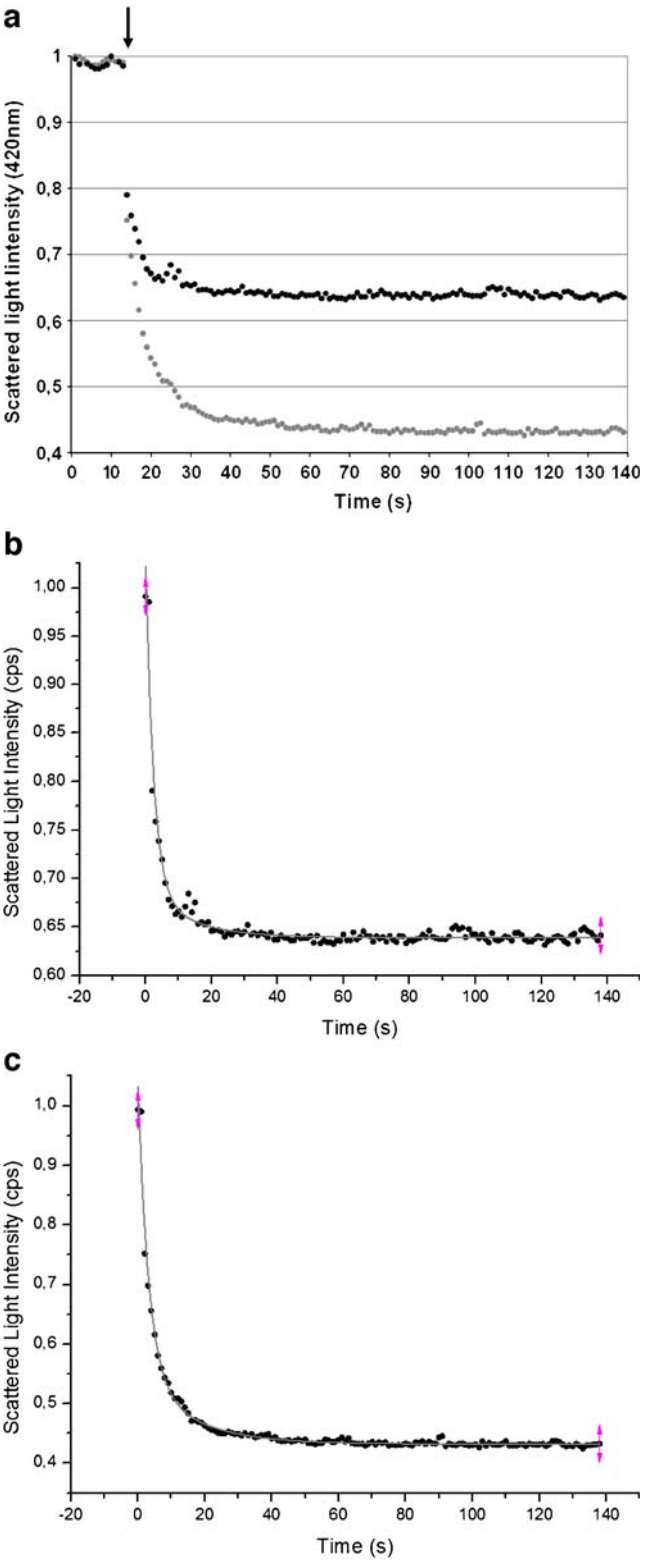


Fig. 4 **a** Effect of the presence of singlet oxygen in the intensity of 420 nm scattered light by a solution of hyaluronic acid labelled with PheSA. Singlet oxygen has been formed upon 420 nm excitation of the photosensitizer TMPyP. The arrow points at the time when the photosensitizer was added. The two data sets simply differ in the amount of sensitizer added to the HA-PheSA solution. The grey dataset corresponds to a sample with twice the concentration of sensitizer compared to the black dataset; **b** Exponential fitting to the data presented in panel A (black data set), with recovered lifetimes and pre-exponential factors displayed in Table 2; **c** Exponential fitting to the data presented in panel A (grey data set), with recovered lifetimes and pre-exponential factors displayed in Table 2

get maximal excitation of the sensitizer TMPyP, leading to singlet oxygen formation. Furthermore, 420 nm light will be efficiently scattered by the solution. The arrow marks the time at which TMPyP has been added. It can be seen that immediately upon TMPyP addition the intensity of light scattered by the HA matrix decays exponentially (Fig. 4a). The two decays displayed differ in the amount of photosensitizer added (twice the amount of TMPyP has been added in the experiment represented by the grey data set). Data has been best fitted by a bi-exponential decay model (Fig. 4b and c). Recovered decay times and pre-exponential factors are listed in Table 2. It can be seen in this table that higher concentration of TMPyP, and therefore of singlet oxygen, has led to a change in the pre-exponential factors associated to each decay time: the fraction of molecules associated with the short decay time (2-3 s) has decreased from 87% to 80% and the fraction of molecules decaying with the longer decay time (13–15 s) has increased from 12% to 20%. In Fig. 5 are displayed the intensity of scattered light of the control samples in the absence and presence of TMPyP. The experiments have been carried out exactly has explained for HA-PheSA. It can be observed that the only sample that suffers changes in the intensity of the scattered light upon addition of TMPyP is HA-PheSA. The intensity of scattered light by all other molecules in the cuvette is unchanged upon the addition of TMPyP. For all samples, the addition of the photosensitizer has been done at the same time after loading the sample and checking for the initial scattered light intensity (see arrows in Fig. 5).

Discussion

The streak camera results presented in this study show that the HA matrix is a strong fluorescence quencher of electronically excited phenyl succinic acid. Furthermore, DLS data shows that the HA matrix is also a scavenger of singlet oxygen, leading to a rapid depolymerisation of HA.

Hyaluronic acid has been derivatised with the anhydride form of phenyl succinic acid (lactone for of phenyl succinic acid). This particular lactone is a 5-membered ring lactone. The most stable structure for lactones are the 5-membered lactones (gamma-lactone) and 6-membered lactones (delta-lactone) because of the minimal angle strain in the compounds' structure. Once attached to the HA matrix,

Table 2 Recovered decay times and pre-exponential factors when fitting the scattered light intensity decays displayed in Fig. 4a

Sample	Lifetimes and pre-exponential factors		Reduced χ^2	R^2
	τ_1 [s]	τ_2 [s]	9,2e-5	0,957
HA-PheSA 4 µl TMPyP	2,4±0,2	13,6±4,9		
	α_1	α_2		
	0,87±0,01	0,13±0,02		
	τ_1 [s]	τ_2 [s]		
HA-PheSA 8 µl TMPyP	3,2±0,2	15,5±0,5	1,1e-4	0,983
	α_1	α_2		
	0,80±0,02	0,20±0,02		

this lactone will open and acquire a carboxylic acid group. As shown in Fig. 1a, both PheSA and HA-PheSA show a characteristic aromatic fluorescence excitation peak at ~280–290 nm. As shown in Fig. 1b, HA-PheSA shows a characteristic aromatic fluorescence emission peak around 350 nm, which is blue shifted compared to the peak observed in PheSA, possibly indicating that the aromatic moiety of PheSA is in a more hydrophobic environment. Time resolved fluorescence data does show that once attached to the HA matrix, the ~350 nm PheSA fluorescence peak is considerably quenched by the HA moiety, which is responsible for the extremely fast fluorescence decay of approximately 20 ps: only a very short fluorescence decay time is observed within 1.8 ns after excitation, as seen in the spectra displayed in Fig. 2a.

Hyaluronic acid is a glycosaminoglycan, being the repeating disaccharide unit of hyaluronan (-4GlcUAβ1-3GlcNAcβ1-)$_n$, as displayed below. It is known that protonated carboxylic acid groups are fluorescence quenchers [25]. Also, possible hydrogen-bonding interaction between PheSA and the hydroxyl and carboxylic acid groups in the HA matrix can contribute to excited singlet state deactivation. It is known that hydrogen bonding

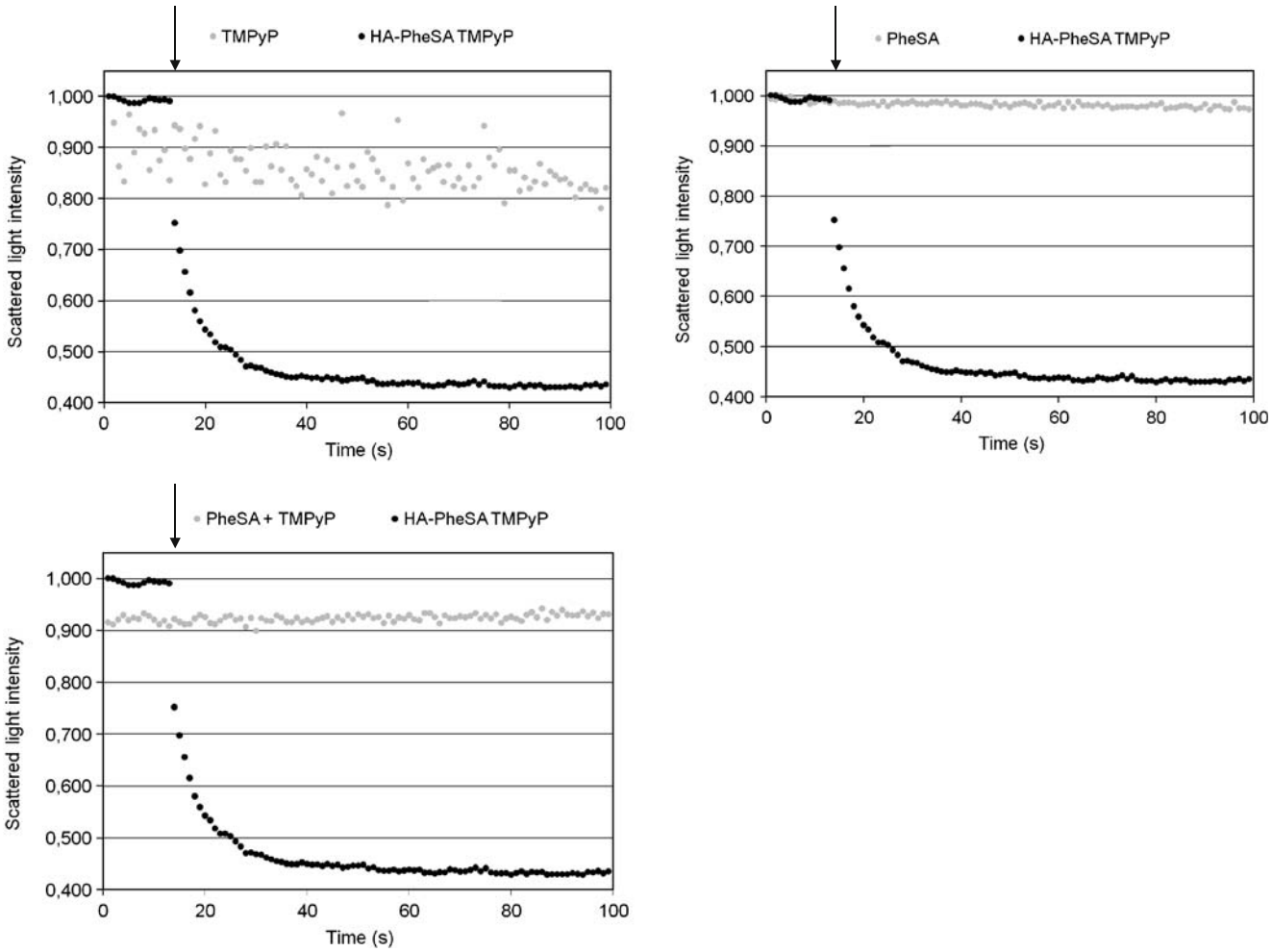
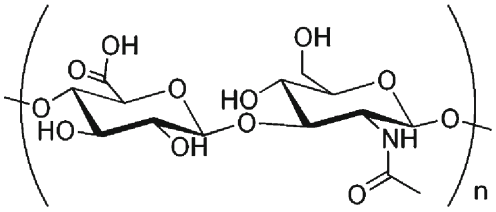
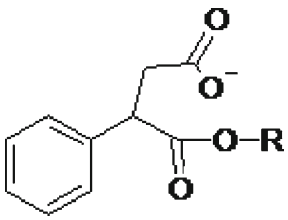


Fig. 5 Control experiments showing the effect of the presence of singlet oxygen in the intensity of 420 nm scattered light by a solution of: hyaluronic acid labelled with PheSA (Phenyl succinic acid anhydride), PheSA and TMPyP, TMPyP, and PheSA. The sensityzer TMPyP has been added to all samples at the same time, as described in Fig. 4

efficiently deactivates singlet excited states of fluorophores [25, 26].



Once derivatised onto the HA matrix, PheSA can form hydrogen bonds between the oxygen atoms in its ester and carboxylic acid groups (see figure below, where R can be any primary or secondary hydroxyl group on the two repeating monosaccharides in the HA structure) and the hydroxyl and carboxylic acid groups in the HA matrix.



Therefore we believe that hydrogen bonding between PheSA and the HA matrix can contribute to the shorter fluorescence lifetime observed in HA-PheSA. Furthermore, when observing Fig. 1b we can see that the characteristic PheSA emission peak around ~420–440 nm is not present when PheSA is linked to HA matrix, which indicated that this peak was correlated to the presence of the lactone ring linked to the phenyl moiety.

The two fluorescence lifetimes observed when PheSA is attached to the matrix possibly indicates that some molecules of PheSA sit in a more quenching environment than others. The molecules embedded in the HA gel matrix would be most likely more quenched due to the presence of protonated carboxylic groups and hydrogen bonding networking as explained above than the PheSA molecules which would be solvent exposed. Furthermore, solvent accessible carboxylic groups would be most likely deprotonated (pK_{model} 4–5), rendering them poorer fluorescence quenchers than the corresponding protonated form. Furthermore, it is observed that the fraction of molecules associated to the longer fluorescence lifetime (248 ps) is very small, and calculations show that after 208 ps the term associated with the shorter lifetime will be less than the term associated with the longer lifetime. For these reasons we judge that the long component is real and necessary for explaining the experimental observables.

When in the presence of singlet oxygen, it was observed not only that the HA matrix depolymerised (Fig. 4a, Table 2) but also that PheSA regained a longer average fluorescence lifetime component after exposure to singlet oxygen (Fig. 3a, Table 1). These two observations might be correlated, since depolymerisation of HA matrix will induce that PheSA no longer is embedded in a fluorescence quenching environment. Data also shows that the larger the amount of singlet oxygen present the larger the depolymerisation of the HA matrix, since the intensity of scattered light dropped to lower values, indicating further depolymerisation of the matrix (Fig. 4a). As mentioned before, smaller particles scatter less efficiently light of the same wavelength than larger particles. The kinetics of depolymerisation of the HA matrix are similar in the two experiments carried out with two different concentrations of singlet oxygen, pointing at the same depolymerisation mechanism. Singlet oxygen will most likely lead to the disruption of particular bonds in the HA matrix, leading to its observed depolymerisation. The larger the extent of HA depolymerisation, the larger the number of PheSA groups that no longer are immersed in the fluorescence quenching matrix, which will give rise to an increase of the average fluorescence lifetime of PheSA (Table 1). Indeed we have observed that the longer the pre-illumination time of TmPyP with 420 nm light, in the presence of HA-PheSA, the longer the average fluorescence lifetime of HA-PheSA solution, indicating that a larger fraction of PheSA groups are no longer attached to the HA matrix (data not shown).

The singlet oxygen scavenging properties of HA and its excited state quenching properties indicate that HA will protect cells and tissues from photophysical and photochemical processes triggered by light and induced by ROS species. The shorter the excited states lifetime the smaller the likelihood for excited state mediated reactions, which could lead cell and tissue damage, especially of excitation involve, e.g., the formation of radical and ionic species and electron transfer. The observed properties of HA make it a pharmaceutical compound with interesting medical properties. It is likely that HA once incorporated into, e.g., solar creams, contribute to the quenching of the excited states of the many aromatic compounds that are part of the solar cream. This way HA will contribute to the prevention of, e.g. radical formation in the cream that could itself lead to cell damage.

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