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Assessment of alginate hydrogel degradation in biological tissue using viscosity-sensitive fluorescent dyes

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Abstract
The main goal of this study is to investigate a combination of viscosity-sensitive and viscosity-insensitive fluorescent dyes to distinguish different rheological states of hydrogel based biostructural materials and carriers in biological tissues and to assess their corresponding location areas. The research is done in the example of alginate hydrogel stained with viscosity-sensitive dyes Seta-470 and Seta-560 as well as the viscosity-insensitive dye Seta-650. These dyes absorb/emit at 469/518, 565/591 and 651/670 nm, respectively. The rheological state of the alginate, the area of the fluorescence signal and the mass of the dense alginate versus the calcium gluconate concentration utilized for alginate gelation were studied in vitro. The most pronounced change in the fluorescence signal area was found at the same concentrations of calcium gluconate (below ~1%) as the change in the alginate plaque mass. The stained alginate was also implanted in situ in rat hip and myocardium and monitored using fluorescence imaging. In summary, our data indicate that the viscosity sensitive dye in combination with the viscosity-insensitive dye allow tracking the biodegradation of the alginate hydrogel and determining the rheological state of hydrogel in biological tissue, which both should have relevance for research and clinical applications. Using this method we estimated the half-life of the dense alginate hydrogel in a rat hip to be in the order of 4 d and about 6–8 d in rat myocardium. The half-life of the dense hydrogel in the myocardium was found to be long enough to prevent aneurysm rupture of the left ventricle wall, one of the more severe complications of the early post-infarction period.

1. Introduction
Biodegradable polymeric hydrogels including alginate polysaccharide hydrogels are used in medicine as surgical implants [1, 2] in particular to prevent heart aneurysm in post-infarction period [3, 4] and to treat advanced chronic heart failure [5, 6], as fillers to eliminate bone defects [7, 8] and as dressings on deep infected wounds [9, 10]. Hydrogel based biomaterials are also used in clinical pharmacology as transport systems [11] for targeted delivery of drugs [12, 13] and cells [14, 15], for immobilization and proliferation of mammalian cells, and as injectable solutions and pastes with delayed gelation [2] but also for many other medical, veterinary and biological applications [16].

The rheological properties (i.e. mechanical resistance and viscoelastic properties) of hydrogels are important parameters for specific biological and medical applications [12] but also for developing new injectable biomaterials along the path of new medical device creation [17]. The stiffness of hydrogels has been reported to be a direct criterion for the differentiation of cell types [18–20]. For drug delivery, hydrogels should preferentially reduce in viscosity upon injection and undergo rapid recovery upon removal of the stress.
to form the drug release gel depot [21]. Rheological measurements allow also for the understanding of the different gelation mechanisms which can be utilized in the optimization of the properties of the hydrogels for tissue reconstruction and drug delivery applications [12]. An important requirement for biocompatible implants is the ability of controlled biodegradation with the formation and removal out of a body the decay products and their gradual replacement with the donor’s tissues [22].

A challenging task is therefore the determination of the rheological properties of hydrogels [23] but also the real time tracking of hydrogel degradation, and the differentiation of the localizations of the dense hydrogel and its biodegradation products formed as a result of the hydrogel decomposition and resorption in biological tissues [24].

The viscoelastic properties of hydrogels can be determined in vitro by rheometry [25, 26] and elastography [27]. The hydrogel structure was explored in vitro using thermo-gravimetric analysis, infrared spectroscopy and scanning electron microscope [28]. Mechanical properties of hydrogels were investigated by tensiometry [29]. To study the biostability of the hydrogels in vitro under close to natural conditions special experimental techniques using the porous cells (beads) of different origins, followed by physico-chemical examining of the contents of microspheres when completing the experiment were applied [30, 31]. Morphological and histochemical techniques were used to help reveal the degradation products in the surrounding hydrogel implant tissues after its administration [32]. Biotin was used for an accurate assessment of the hydrogel distribution and degradation, which was added before cross-linking of alginate with calcium [4]. In a number of the studies 14C radio-active labeling [33], radioioditation [34] and 1H-NMR microimaging were used to evaluate biomaterial degradation [35], while optical coherence tomography was applied for the direct determination of biodegradable hydrogel parameters in microspheres and implants [36].

To track hydrogels in vivo in biological tissues highly-sensitive fluorescent methods can be used. In general, these methods are more sensitive compared to the non-fluorescent methods because they require only 10⁻⁶–10⁻⁸ M of a fluorescent reporter. Thus a non-invasive in vivo monitoring of biodegradable gelatin hydrogel, non-covalently labeled with fluorescent meso-brominated pentamethine cyanine dyes, was recently reported [37]. The non-invasive in vivo monitoring of PEG—dextran hydrogels and collagen was realized using Texas Red and fluorescein, covalently linked to hydrogel molecules [38]. The fluorescence based approach was used for the non-invasive determination of chitosan membranes covalently labeled to tetramethylrhodamine isothiocyanate (TRITC) [39], collagen covalently labeled with cyanine dye ZW800-1 [40], PEGLated fibrinogen covalently bound to cyanine dye Cy5.5 [1], and alginate hydrogel covalently labeled with fluorescein isothiocyanate (FITC) [41, 42] or rhodamine B isothiocyanate (RITC) [42]. Bovine serum albumin (BSA) fluorescently labeled with FITC was used to simulate drug release from alginate composite hydrogel [43].

Importantly, all the investigated dyes were environment-insensitive i.e. their spectral characteristics, such as emission spectrum and intensity were insensitive to viscosity, polarity or hydrophilicity changes in the medium. Therefore these dyes did not enable the characterization of the rheological properties of the hydrogel and/or to distinguish the fluorescence signal from the dye bound to: (i) highly-viscous, non-degraded dense hydrogel, (ii) lower-viscous degrading hydrogel and degradation products, and (iii) free dye eliminated from degrading hydrogel—because all these species have about the same fluorescence characteristics.

The objective of this study was to explore the viscosity-sensitive dyes Seta-470 and Seta-560 in combination with a viscosity-insensitive dye Seta-650 to differentiate between the dense alginate hydrogel and its low-viscous degradation products in a muscle tissue model—rat hip and myocardium, which is in particular important for clinical applications of alginate hydrogel as a biodegradable implant and targeted drug delivery carrier.

2. Experimental

2.1. Animal experiments

The experiments were performed on 76 Wistar male rats weighing 180–250 g. The research subject was superficial gluteal muscles (m. gluteus superficialis) and myocardium.

Animal experiments were done in the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. All procedures involving the use and care of animals conform to the ‘Guide for the Care and Use of Laboratory Animals’ published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by Stockholm Southern Ethics Review Board. The experimental protocols were approved by the Commission in Bioethics of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine.

Animals were maintained at standard animal house conditions of 3 daily meals at +20 °C air temperature and 65% humidity. Intraperitoneal ketamine anesthesia was applied in a dose of 75 mg kg⁻¹. For additional analgesia the nalbuphine was intramuscularly administered in 1 mg kg⁻¹ dose. The animals were removed from the experiments by anesthetic overdosage.

2.2. Materials

Sodium alginate (medium viscosity, MW ~ 350 000 Da [44]) was from Sigma (A2033). Amine-reactive fluorescent dyes Seta-470-NHS, Seta-560-NHS and Seta-650-NHS were from SETA BioMedicals, Urbana, Il (USA), 4-Morpholineethanesulfonic acid, N-(3-dimetylaminopropyl)-N’-ethylcarbodiimide
hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 1,6-diaminohexane were from Aldrich. Solvents were from Merck. All starting materials were used without further purification as purchased.

2.3. Preparation of amino-modified sodium alginate

2.0 g of sodium alginate (Alg) was dissolved in 200 ml of 50 mM MES buffer pH 6.1. MES buffer was prepared by dissolving 9.8 g of 4-morpholineethanesulfonic acid in 11 water and then the pH was adjusted to pH 6.1 using concentrated sodium hydroxide. 930 mg of EDC and 310 mg of NHS were added under constant stirring to the solution of alginate. After 30 min, 2 g of 1,6-diaminohexane were added to the reaction mixture and stirred for 12 h. To remove residual 1,6-diaminohexane, the resulted amino-modified sodium alginate was precipitated with isopropanol, filtered, washed with isopropanol, and dried. Yield: 2.03 g.

2.4. Preparation of sodium alginate fluorescently labeled with dyes Seta-470 and Seta-650

To a solution of 270 mg of amino-modified sodium alginate in 15 ml of 50 mM bicarbonate buffer pH 9.0 was added a solution of 6.0 mg of Seta-470-NHS ester and 5.0 mg of Seta-650-NHS ester in 1 ml of N,N-dimethylformamide (DMF). The resulting solution was stirred for 2 h at room temperature. To remove residual non-conjugated dyes, the resulted alginate—dye conjugate was precipitated with 50 ml of isopropanol, filtered, washed with isopropanol, and dried. Yield of Seta-470—Alg: 215 mg. The molar ratio of the dyes and alginate (Seta-470): (Seta-650): (Alginate) (1.3 : 1 : 2.5) and the degree of functionalization were calculated using the absorbances measured for the pre-weighted samples of stained alginate and the Beer–Lambert–Bouguer law. The molar absorptivities (ε) were 50 000 M⁻¹ cm⁻¹ (Seta-470) and 200 000 M⁻¹ cm⁻¹ (Seta-650). λmax Abs. = 469, 651 nm; λmax Em. = 518, 671 nm (measured in water, λex. = 530 nm). The obtained alginate—dye conjugate did not contain physically trapped dye, which was confirmed by the size-exclusion chromatography (Sephadex G-50, 67 mM phosphate buffer, pH7.4).

2.5. Preparation of sodium alginate fluorescently labeled with dyes Seta-560 and Seta-650

To a solution of 150 mg of amino-modified sodium alginate in 8.5 ml of 50 mM bicarbonate buffer pH 9.0 was added a solution of 2.5 mg of Seta-560-NHS ester and 2.7 mg of Seta-650-NHS ester in 0.5 ml DMF. The resulting solution was stirred for 3 h at room temperature. To remove residual non-conjugated dyes, the resulted alginate—dye conjugate was precipitated with 30 ml of isopropanol, filtered, washed with isopropanol, and dried. Yield of Seta-560—Seta-650—Alg: 149 mg. The molar ratio of the dyes and alginate (Seta-560): (Seta-650): (Alginate) (2.5 : 1 : 4.2) and the degree of functionalization were calculated using the absorbances measured for the pre-weighted samples of stained alginate and the Beer–Lambert–Bouguer law. The molar absorptivities (ε) were 65 000 M⁻¹ cm⁻¹ (Seta-560) and 200 000 M⁻¹ cm⁻¹ (Seta-650). λmax Abs. = 565, 651 nm; λmax Em. = 591, 669 nm (measured in water, λex. = 530 nm). The obtained alginate—dye conjugate did not contain physically trapped dye, which was confirmed by the size-exclusion chromatography (Sephadex G-50, 67 mM phosphate buffer, pH7.4).

2.6. Implantation of fluorescently labeled alginate hydrogel into rat hip muscle

The animal, which had undergone ketamine anesthesia was placed on a surgical table. After pretreatment of the surgical area (shaving hair and antiseptic treatment) a longitudinal incision through rat skin was made and the operating wound was extended using holders.

Then 0.04 ml of 10% calcium gluconate solution (CaGluc) and 0.05 ml of 1.0–1.5% fluorescently labeled sodium alginate in normal saline solution were consistently filled up in a syringe without agitation. A needle with a 20° bevel tip was inserted at a ~20° angle into the m. gluteus superficialis (heel up) and the entire solution in the syringe was injected to a required depth of 1 mm. To prevent leakage of the injected solution, the needle was removed from the muscle with a delay of 4–5 s after the injection. The interaction of sodium alginate with calcium gluconate in the muscle tissue results in situ formation of fluorescently labeled, dense alginate implant. Subsequently the fluorescence images were taken at time point zero and the wound was sutured.

An opposite limb was intramuscularly injected with nalbuphine of 1 mg kg⁻¹ of body weight. The rat was placed into the recovery room. For re-measurement of the fluorescent alginate hydrogel images the rats were administered with anesthesia and skin sutures were revealed before each measurement. After photographing the wound was treated with an antiseptic (0.5% chlorhexidine in 70% aqu. ethanol) and the skin was re-sutured.

2.7. Implantation of fluorescently labeled alginate hydrogel into rat myocardium

The rat which had undergone ketamine anesthesia was fixed on a surgical table. After pre-treatment of a surgical field (shaving hair and antiseptic treatment) the thoracotomy in 4–5 intercostal space was performed. Heart of live rat was exteriorized through a surgical wound.

Simultaneously with the surgery, 0.03 ml of 10% calcium gluconate solution and 0.04 ml of 0.5–1% fluorescently labeled sodium alginate in normal saline solution were consistently filled up in a syringe without agitation. A needle with 20° bevel tip was inserted at a ~20° angle into left ventricular myocardium and the entire solution in the syringe was rapidly injected to a required depth of 1 mm. The injection was done immediately after the heart was deduced. To prevent leakage of the injected solution, the needle was removed from the wound.
myocardium with a delay of 4–5 s after the injection. Interaction of sodium alginate with calcium gluconate in tissue results in the in situ formation of fluorescently labeled, dense alginate implant.

Then the heart was removed to get a zero time point or returned back into the chest cavity to get the next time point and the wound was layer-by-layer sutured. For an animal’s anesthesia nalbuphine was intramuscularly injected into a rat hip in a dose of 1 mg kg\(^{-1}\) of body weight. At certain time after gel injection the experimental animals were euthanized with a ketamine overdose, the hearts were removed and frozen for further imaging.

### 2.8. Spectral measurements

Absorption and emission spectra were measured in normal saline solutions, water, methanol, and glycerol at 25 °C in 1 cm standard quartz cells. The absorption spectra were recorded using a PerkinElmer Lambda 35 spectrophotometer and the emission spectra were taken using a Varian Cary Eclipse spectrofluorimeter. The emission spectra were corrected. The absolute fluorescence quantum yields for Seta-470, Seta-560 and Seta-650 were measured using fluorescein in 0.1 M aqueous NaOH solution (91%) [45, 46], rhodamine B in methanol (82%) [47] and Cy5 in phosphate buffer pH 7.4 (27%) [48] as standards, respectively.

### 2.9. Fluorescence imaging in vitro

To obtain in vitro images of fluorescently labeled alginate at different calcium gluconate concentrations (c(CaGluc)), a series of CaGluc solutions (10, 8, 6, 4, 2, 1, 0.5, 0.25 and 0.1%) in normal saline were prepared. Then the two samples of 0.02 ml of 1.0% fluorescently labeled sodium alginate in normal saline solution were placed on Petri dishes and 0.015 ml of calcium gluconate solution (CaGluc) of each concentration were added into the drop of sodium alginate solution, mixed gently with the syringe needle and Petri dishes were covered with the glass lids.

To avoid dependence of the measured fluorescence intensities and areas versus the excitation power, filters, dye brightness, and other experimental conditions, standards of brightness were used in the imaging experiments. These standards were small pieces (3–4 mm diameter) of the fluorescently labeled, dense alginate.

Then the lids were removed from Petri dishes and the images were obtained as described below. The images were taken in 10 and 30 min and after 1 h of gel preparation and these images were found unchanged during this time frame. The images taken in 30 min are presented in the paper. Four images for each c(CaGluc) were obtained, mathematically processed as described below and the average values of the calculated bright fluorescence areas were plotted as a function of c(CaGluc).

### 2.10. Fluorescence imaging in vivo

A small sample (3–4 mm diameter) of the same fluorescently labeled, dense alginate that was introduced into the rat hip or myocardium was placed on a black ruler at a small distance (1–3 cm) from the injected alginate and used as a brightness standard. Images of the biological sample containing the injected hydrogel were taken as described below at specific time periods after injection.

The fluorescence signal from the sample (rat hip or heart) was obtained via irradiation with an LED through a bandpass filter at a distance of 10 cm. A long-pass filter located in front of the camera lens was used to cutoff the excitation light. In case of the red-excitable, red-emitting dye Seta-650 (red channel) a 636 nm 3 W LED, a 640/10 nm bandpass excitation filter and a 670 nm longpass emission filter were used. For the green-excitable, orange-emitting dye Seta-560 (orange channel) a 530 nm 3 W LED, a 530/20 nm bandpass excitation filter and a 600 nm longpass emission filter were used. For the blue-excitable, green-emitting dye Seta-470 (green channel) a 470 nm 3 W LED, a 470/10 nm bandpass excitation filter and a 546/20 nm bandpass emission filter were applied. Fluorescence images were recorded using a color digital camera NIKON D610 at the constant matrix sensitivity (ISO-400), constant focus distance (85 mm), constant aperture, and constant exposure (4 s for red and orange channels and 20 s for green channel).

To register signal from the orange-emitting dye Seta-560 (‘orange’ channel) we used a 600 nm longpass emission filter and therefore the images obtained in the ‘orange’ channel are red. This filter was used to better separate fluorescence signal of the orange-emitting dye Seta-560 from the scattered excitation light 530 nm. The images were obtained by a color camera and are presented in the paper without color correction. Therefore the images obtained in both orange and red canals are red.

The fluorescence images obtained in each channel were adjusted in such a way that the standards of brightness on these images had equal brightness. Then the alginate fluorescence areas were localized and quantified as described below.

### 2.11. Alginate observation in rat hip

Experimental animal under ketamine anesthesia was fixed on a surgical table. After pretreatment of the surgical field (shaving hair and antisepctic treatment) the postoperative wound was extended using holders. The fluorescence images were taken in normal light and then in the green and red channels for Seta-470—Seta-650—Alg or orange and red channels for Seta-560—Seta-650—Alg and the edges of the wound were sutured.

### 2.12. Alginate observation in rat myocardium

The location of the gel implant in each heart was found visually by its fluorescence signal obtained in the red channel. Then the heart was cut at the place of the maximal concentration of the fluorescing implant and the images were taken in the green and red channels for Seta-470—Seta-650—Alg or orange and red channels for Seta-560—Seta-650—Alg.
2.13. Measurements of hydrogel mass versus the calcium gluconate concentration in vitro

A set of 1.5 ml calcium gluconate solutions in normal saline with concentrations \( c(\text{CaGlu}) = 10\% \), 8\%, 6\%, 4\%, 2\%, 1\%, 0.5\%, 0.35\%, 0.25\%, and 0.1\% were prepared. To these solutions was added 2 ml of 1\% solution of Seta-470—Seta-650—Alg. The final concentrations of calcium gluconate were 4.29\%, 3.44\%, 2.58\%, 1.72\%, 0.86\%, 0.43\%, 0.21\%, 0.15\%, 0.11\%, 0.04\%, and 0\%, respectively. The obtained mixtures were shaken for 1 min and left for 30 min for gel formation. Then the mixtures were filtered through a paper filter (Whatman® quantitative filter paper, ashless, Grade 40) for 1 min with sucking at 30 mbar. Only in the concentration range of CaGlu between 0.86–4.29\% gel plagues were obtained and their masses were measured without drying. Four experiments for each \( c(\text{CaGlu}) \) were done, statistically processed and the average values of the gel masses were plotted as a function of \( c(\text{CaGlu}) \).

2.14. Histological and morphometric analyses

Postmortem morphometric analysis was performed on rat hearts in 1 h and on the 38th day after alginate injection. The hearts were arrested with 15% KCl and the left ventricle was sectioned into longitudinal slices. Each slice was fixed with 10% buffered formalin, embedded in paraffin, and sectioned into 5 \( \mu \)m slices. The slides were stained with hematoxylin and eosin (H&E), studied by light microscopy with microscope ‘Granum R 4003’ and analyzed with software (BioVision, Version 4.0) for image analysis.

2.15. Image processing and statistical analysis

All the images had the same size (2784 × 1856 pixels). The images were processed and analyzed using ImageJ software available from the United States National Institute of Health (http://rsbweb.nih.gov/ij/index.html). Fluorescence areas and the integrated intensities (brightness) of the samples and the standards of brightness were localized using the ‘Wang (tracing) tool’ (mode Legacy, Tolerance 25) and determined using the ‘Analyze—Measure’ command. The result of this command is ‘Area’ and ‘Integrated Density’. Then the calculated fluorescence areas and integrated density of the samples were divided by the same values determined for the standards of brightness to make them independent on the possible deviations in the measurement conditions such as distance between the camera and the sample, intensity of the excitation light, camera sensitivity, exposure, etc. The obtained areas were normalized by 100\% and corresponding plots were drown.

The half-lives of the dense hydrogel were calculated from the fluorescence area decrease profiles obtained with dyes Seta-470 and Seta-560. The two-exponential fit was used for Seta-470 and mono-exponential fit for Seta-560.

The four imaging experiments were done for each data point (\( n = 4 \)). All of them were processed as described above and a statistical analysis was used to get the average data.

Statistical analysis was carried out using a one-way ANOVA. Values were considered to be statistically significant at a value of \( p \leq 0.05 \). Data on the plots are presented as mean ± SD.

3. Results and discussion

3.1. Spectral characterization of dyes free in solution and when bound to dense and non-gelated alginate

The fluorescent dyes Seta-470, Seta-560, and Seta-650 which absorb/emit in the blue/green, green/orange, and red/red regions, respectively, were used as reporters to monitor alginate in tissue (figures 1(a)–(c)). The distinct excitation and emission spectra of these dyes enable separate registration of their fluorescence signals (green, orange and red channels) using appropriate excitation light sources, excitation and emission filter sets.

First the sensitivity of the dyes to the viscosity of the medium by comparing the spectral properties of dyes Seta-470, Seta-560, and Seta-650 bound to a non-gelated alginate and those in a normal saline solution was estimated. The data show that the fluorescence intensities of Seta-470 and Seta-560 increase almost twice upon binding to a non-gelated sodium alginate (figures 1(a) and (b)). At the same time these characteristics for Seta-650 remain almost unchanged (figure 1(c)).

Most importantly the fluorescence intensities of Seta-470 and Seta-560 are sensitive only to the viscosity and insensitive to the polarity and hydrophilicity of the medium. Thus the fluorescence quantum yield of Seta-470 in water (1.9\%) was found to be about the same as in methanol (2.0\%) a solvent with much lower polarity while it dramatically increases up to 40% in glycerol which has similar polarity as methanol (0.812 versus 0.764) [49] but much higher viscosity—1400 mPa × s in glycerol versus 0.6 mPa × s in methanol. A similar behavior was found for Seta-560: the quantum yields are 2.3%, 1.7% and 50% in water, methanol and glycerol, respectively.

It is worth mentioning that the longer wavelength red/NIR light much easier penetrates through biological tissue as compared to the shorter wavelength blue, green and orange ones [30]. Therefore the molar ratios of the dyes (Seta-470) : (Seta-650) and (Seta-470) : (Seta-650) bound to alginate (Alg) were adjusted in such a way that the fluorescence signals of these dyes in biological tissue were in the same order. Alginate with the molar ratio (Seta-470) : (Seta-650) = 1.3 : 1 and (Seta-560) : (Seta-650) = 2.5 : 1 were elected for further experiments. The absorption and emission spectra of the dyes when bound to non-gelated alginate at the molar ratio (Seta-470) : (Seta-650) = 1 : 1
and \((\text{Seta-560}) : (\text{Seta-650}) = 2.5 : 1\) are represented in figures 1(d) and (e), respectively. The excitation wavelength was 470 nm for \(\text{Seta-470} - \text{Seta-650} - \text{Alg}\) and 530 nm for \(\text{Seta-560} - \text{Seta-650} - \text{Alg}\).

Then the correlation between the emission intensities of the stained alginates and the calcium gluconate concentration, \(c(\text{CaGluc})\), were explored. The fluorescence images of the stained alginates containing different amount of CaGluc were taken (figure 2) and the fluorescence intensities were measured. Addition of CaGluc causes alginate gelation, resulting in a dramatic increase in viscosity. The fluorescence intensities of viscosity-sensitive dyes \(\text{Seta-470}\) and \(\text{Seta-560}\) were found to increase by a factor of 5 and 3, respectively, while for \(\text{Seta-650}\) the increase was only by factor of 1.3.

Then the bright fluorescence areas of alginate were determined. Importantly, the bright area of the dense alginate stained with \(\text{Seta-470}\) or \(\text{Seta-560}\) dramatically increases upon increasing the concentration of CaGluc (figures 2 and 3), which indicates the sensitivity of these dyes to the alginate viscosity. In contrast, the fluorescence area of the viscosity-insensitive dye \(\text{Seta-650}\) has a tendency to decrease (by about 15%) because the volume of the alginate slightly decreases when gelated.

The above data evidence that the viscosity-insensitive dye \(\text{Seta-650}\) is capable to detect the locations of the non-gelated alginate, the dense alginate hydrogel and its fluorescently labeled degradation products independent of their rheological state. \(\text{Seta-650}\) does therefore not allow to differentiate between the dense hydrogel and its degradation products. In contrast, the viscosity-sensitive dyes \(\text{Seta-470}\) and \(\text{Seta-560}\) help to localize these dense hydrogel areas and in combination with \(\text{Seta-650}\) allow distinguishing it from the low-viscous fluorescent products of hydrogel degradation.

Biodegradation of alginate hydrogel in biological tissues is connected with the reduction of \(\text{Ca}^{2+}\) concentration due to release of \(\text{Ca}^{2+}\) ions cross-linking the gel into the surrounding media due to blood circulation and exchange reactions with monovalent cations.
such as sodium ions [51]. This process can be modeled in vitro using the alginate hydrogel mass, rheological state and/or viscosity as a function of the calcium gluconate concentration. Therefore the above data on the fluorescence area of alginate versus $c(CaGluc)$ were correlated with the gelated alginate mass and the rheological properties of alginate (friable or dense plaques or non-gelated liquid).

Addition of 4.3% of calcium gluconate to the sodium alginate solution causes formation of the dense (tight) hydrogel plaques which can be separated from the solution by filtration. Decrease in the calcium gluconate concentration from 4.3% to about 0.8% was found to do not affect the mass and rheological state of the plaques (figure 3). Remarkably, further decrease of $c(CaGlu)$ to about 0.2% causes a 2-fold increase in the plaque mass up to about 200% and noticeable decrease in the plaque viscosity, which can be easily identified by eye (the plaques become less tight and more friable). The increase in the plaque mass above 100% of their initial mass is connected with the fact that at $c(CaGlu)$ between ~0.8–0.15% the plaques are very friable and contain more water then at $c(CaGlu) > 0.8%$. Further decrease of $c(CaGlu)$ below ~0.2% results in a dramatic decrease in the plaque mass and then the plaques totally disappear.

<table>
<thead>
<tr>
<th>$c(CaGluc)$ [%] (Rheological state of hydrogel)</th>
<th>Red Channel (Seta-650)</th>
<th>Green Channel (Seta-470)</th>
<th>Orange Channel (Seta-560)</th>
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<tr>
<td>0 (Non-gelated)</td>
<td>![Image]</td>
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<tr>
<td>0.86 (Partially gelated)</td>
<td>![Image]</td>
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<tr>
<td>4.3 (Dense)</td>
<td>![Image]</td>
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Figure 2. Representative in vitro images of the fluorescently stained alginate (in vitro) at different concentration of calcium gluconate.

Figure 3. Fluorescence area (localization) of alginate stained with Seta-470, Seta-560 and Seta-650 and the normalized hydrogel mass versus concentration of calcium gluconate. Values are given as mean ± SD, $n = 4$ at each data point.
Importantly, the most pronounced change in the fluorescence areas was found at the same concentrations of CaGluc (below ~1%) as the change in the alginate plaque mass (figure 3).

### 3.2. Assessment of Rheological Properties and Localization of Alginate Hydrogel in Rat Hip

Gelated alginate hydrogel, fluorescently labeled with dyes Seta-470 and Seta-650 (Seta-470—Seta-650—Alg), was implanted in situ to a depth of 1 mm in the right hips of four (n = 4) live rats and Seta-560—Seta-650—Alg was also delivered to the same depth in the right hips of other four rats (n = 4). Then the images were taken in 10–20 min after implantation and in certain time during 12 weeks. All the images were obtained in normal light and in fluorescence regime in the red channel (λEx = 636/10 nm; λReg > 670 nm). In case of the first alginate the images were taken also in the green channel (λEx = 470/10 nm; λReg = 546/20 nm) and for the second alginate—in the orange channel (λEx = 530/10 nm; λReg > 600 nm). The use of different excitation light sources and filter sets allowed distinguishing the fluorescence signals originated from Seta-470 (green channel) and Seta-650 (red channel) for the first alginate and Seta-560 (orange channel) and Seta-650 (red channel) for the second alginate. Then the emission areas obtained in these three channels were separately quantified. The representative images are given on figure 4 while the plots showing the change of the fluorescence areas in time are given on figure 5.

The obtained fluorescence images exhibit a dramatic decrease in the fluorescence area of Seta-470 and Seta-650 within the 1st day after the alginate hydrogel is implanted in rat hip. For the 1st day the fluorescence area of Seta-650 decreases by factor of 3 and then it remains almost constant during at least 12 weeks (figures 5(a) and (b)). At the same time the emission intensity of Seta-470 becomes lower than the detection limit used in these experiments within 3 d after implantation (figure 5(a)).

Because the alginate hydrogel degradation does not affect the fluorescence intensity of Seta-650, the above mentioned dramatic decrease in the fluorescence areas of Seta-650 and Seta-470 within the 1st day cannot be associated with hydrogel degradation. More likely the decrease is connected with muscle mobility of rat, which causes an immersion of the dense hydrogel deeper into the muscle tissue and removal of non-gelated and non-resorbed alginate with blood stream. The immersion effect can be seen even by eye in normal light: after alginate injection there is a convex at the injection zone, which disappears by the next day.

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**Figure 4.** Representative images of the live rat hips with implanted alginates Seta-470—Seta-650—Alg and Seta-560—Seta-650—Alg. The images were taken in the red, green, and orange channels in 15 min and 3 weeks after implantation.

**Figure 5.** Fluorescence areas (localizations) of alginates Seta-470—Seta-650—Alg (a) and Seta-560—Seta-650—Alg (b) implanted in rat hip versus time. Inserts show 10 d scale. Values are given as mean ± SD, n = 4 at each data point.
Because the red light easier penetrates through tissue, within the 1st day the fluorescence area of Seta-650 reduces due to the gel immersion only by factor of 3 while for the shorter-wavelength dye Seta-470 the fluorescence signal becomes almost undetectable.

Similar to Seta-470 the fluorescence signal of longer-wavelength dye Seta-560 also noticeably decreases but it is still well-defined even within 3 weeks after injection (figure 5(b)), which evidences that the dense alginate is still present in the implantation zone. The half-life ($\tau_{1/2}$) of the dense hydrogel in rat hip was estimated from the decrease in fluorescence area shown in figure 5. The determined $\tau_{1/2}$ is rather apparent but not the real half-life of the dense hydrogel because the fluorescence decrease profiles reflect also the alginate immersion. The apparent $\tau_{1/2}$ was found to be about 2.5 d when estimated using Seta-560 (figure 5(b)) but only 0.5 d when defined using Seta-470 (figure 5(a)). These half-lives were calculated as an average of four experiments using Seta-470 and four experiments using Seta-560. Such a contradiction in the half-lives is connected with the fact that the decrease in fluorescence intensity, estimated using the viscosity-sensitive dyes, is affected not only by gel degradation but it is also due to the gel immersion. As compared to the degradation process, in case of the immersion the observed decrease of the intensity rate is strongly dependent on the excitation and emission wavelength. Seta-560 is excited and emits light at the longer wavelengths as compared to Seta-470 and therefore the real half-life of the dense hydrogel in rat hip should be much longer then apparent half-life of 0.5 d estimated with Seta-470 and even longer then 2.5 d estimated with Seta-560. Taking the immersion of the dense hydrogel into account, its real half-life was estimated to be about 4 d.

While the fluorescence area of the dense alginate stained with Seta-560 becomes almost undetectable within 25–30 d after implantation and within 3 d for Seta-470, the fluorescence area of Seta-650 remains almost unchanged starting from the 2nd day and at least during 12 weeks after implantation (figures 5(a) and (b)). The horizontal section of the curve evidences that the stained alginate is not immersing, not resorbing, and not removing anymore during this time period.

3.3. Assessment of rheological properties and localization of alginate hydrogel in rat myocardium

The same fluorescently stained, dense alginates Seta-470—Seta-650—Alg and Seta-560—Seta-650—Alg were delivered in situ to a depth of 1 mm in myocardium of left ventricular of live rats ($n = 32$ and $n = 36$ for each alginate, respectively). The rat hearts were isolated immediately after delivery and in certain time within 12 weeks, frozen, cut in place of the maximal concentration of the implant and the images were taken and processed as was described for the rat hips. The time-dependent decrease profiles of the fluorescence areas measured in the red (Seta-650), orange (Seta-560) and green (Seta-470) channels are represented on figure 6.

In contrast to the rat hip no dramatic decrease in the emission area measured in all three channels was observed in the 1st day after alginate implantation in rat myocardium (figure 6). Within about 21 d there is only a slight (~15%) monotonic decrease of the alginate area stained with Seta-650, indicating a moderate alginate resorption. Simultaneously, the fluorescence areas of Seta-470 and Seta-560 substantially decrease and become almost undetectable by about the 38th day, while the fluorescence of Seta-650 remains in the implantation zone for at least 12 weeks. The obtained data evidence, that the half-life ($\tau_{1/2}$) of the dense hydrogel in myocardium is about 6–8 d. These half-lives were calculated from the decrease of the fluorescence area shown in figure 6. The half-life of 6 d was calculated as an average of four experiments using Seta-470 and the half-life of 8 d was obtained as an average of four experiments with Seta-560.

The obtained half-life was found to be in a good agreement with observation [32] showing that within 7 d about 60% of the initial alginate hydrogel mass was lost and $\tau_{1/2} \approx 6$ d, which was measured by the dry weight loss over time in vitro and hematoxylin–eosin staining of the frozen rat heart sections.

The half-lives obtained by fluorescence imaging of alginate hydrogel implanted in rat myocardium also correlate well with our own histological data. Histological analysis evidences that the alginate plaque formed within 1 h after injection has distinct shape and a size of about 0.225 mm$^2$ (figure 7(a)). Within 38 d the size of the plaque becomes about 0.75 mm$^2$ (figure 7(b)).
of the plaque significantly reduces to about 0.010 mm$^2$ (figure 7(b)). The decrease found from the histograms (~23 fold) is in good agreement with that measured using dyes Seta-470 and Seta-560 (~15–17 fold).

When forming an extensive acute myocardial infarction the probability of developing heart aneurysm is known to increase at early post-infarction period of up to 4–7 d [52, 53]. The findings on the dense hydrogel half-life in the rat myocardium to days 6–8 in the norm gives evidence to believe that the presence of a viscous alginate implant within this time will prevent the development of left ventricular wall rupture in the post-infarction period. This time is sufficient for eliminating the necrotic myocardium and the newly formed connective tissue to emerge. Passing the 7 d critical threshold, the implant prevents the development of myocardial infarction complications and participates in the formation of connective tissue scar [54].

3.4. Fluorescence profiles

To minimize the dependence of the obtained fluorescence areas versus the measurement conditions, the fluorescence area profiles presented in figures 5 and 6 are normalized by the initial fluorescence areas. Therefore although the initial fluorescence areas presented in figure 4 for the red, orange and green channels are the same (100%), they actually have different ‘absolute’ values.

As seen from figures 5 and 6, alginate hydrogel has different fluorescence area profiles in the rat hip and myocardium when stained with dyes Seta-470, Seta-560 and Seta-650. The first remarkable discrepancy in these profiles is that the fluorescence area of Seta-650 remains almost unchanged between 3 weeks and at least 12 weeks of observation in both rat hip and myocardium. This ‘residual’ fluorescence in the order of about 25% of its initial value for rat hip and ~90% for rat myocardium is reproducible and confirmed by a number of experiments.

More likely this effect is due to the stained alginate is not fully removed from the injection zone, even if it dissolves (changes its rheological state from a dense gel to a liquid). To understand this fact, it is worth mentioning that the polymeric alginate chains cannot be chemically destructed (cleaved) in mammals into the shorter chains or monomers, as mammals lack the enzyme (i.e. alginase) which can cleave the polymer chains [34]. Neither hydrolytic nor enzymatic chain breakages occur within alginate chains under physiological conditions. Subcutaneous injected sodium alginate solution was reported to retain in the site of injection in quantities of 70% at 24 h. I onically cross-linked alginate gels can be dissolved by release of the divalent ions cross-linking the gel into the surrounding media due to exchange reactions with monovalent cations such as sodium ions. Even if the gel dissolves, its average molecular weight (~350 000 Da) does not change and it is much higher than the renal clearance threshold of the kidneys (~48 000 Da). As a result regardless of its rheological state alginate cannot be completely removed from the body [34]. Therefore after the gel has been degraded, the fluorescently stained alginate is not fully removed from the injection zone and the fluorescence signal of Seta-650 remains unchanged on a certain level (~25% for rat hip and ~90% for rat myocardium) while the fluorescence signals of viscosity sensitive dyes Seta-470 and Seta-560 dramatically decrease. Alginate hydrogel injected in relatively thick rat hip immerses deeper in tissue and as a result the fluorescence signal reduces by 75% (from 100% to 25%). In contrast, rat myocardium has a thickness of about 2.0–2.5 mm in the injection zone and therefore the stained alginate cannot immerse and the fluorescence signal reduces therefore only by 10% (from 100% to 90%).

The second important observation is that while the fluorescence signal of Seta-650 is still well-defined during 12 weeks and there are no signs of its further decrease, the signal of Seta-560 in rat hip becomes undetectable in about 25 d and in 1–2 d for Seta-470. The difference in the fluorescence area profiles between Seta-650 from one side and Seta-470 and Seta-560 on the other side is due to the last ones are more sensitive...
to the alginic rheological state and immersion. Seta-560 shows a more uniform fluorescence area profile and longer half-life as compared to Seta-470 because it is excited and emits light at longer wavelengths and therefore is less sensitive to immersion.

In rat myocardium Seta-470 and Seta-560 exhibit different fluorescent area profiles as in rat hip; they are more uniform and the fluorescence is still detectable even after 35 d after injection. This is due to rat myocardium has a thickness in the injection zone of about 2.0–2.5 mm and the stained alginic (both gel and liquid) cannot immerse. Therefore, the area profile reflects only the change in the rheological state and resorption. Nevertheless Seta-560 shows a longer half-life (8 d) as compared to Seta-470 (6 d), which is due to its longer-wavelength absorption and emission.

4. Conclusions

The unique properties of two viscosity-sensitive fluorescent dyes, Seta-470 and Seta-560 to track the biodegradation of the dense alginic hydrogel (implant) delivered in situ in muscle tissue and to determine the rheological state of the hydrogel, both of research and clinical relevance, were demonstrated. Using these dyes the half-life of the dense alginic hydrogel in rat hip was found to be about 4 d and about 6–8 d in rat myocardium. The dense hydrogel half-life in myocardium is sufficient to prevent the aneurysm rupture of the left ventricle wall, one of the severe complications at early post-infarction period. Using the viscosity-insensitive dye Seta-650 it was demonstrated that the degraded high-molecular weight alginic (MW ~ 350 000 Da) remains in the implantation zone of the rat hip and myocardium for at least 12 weeks.

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Competing interests

The authors have declared that no competing interests exist.

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