J Fluoresc (2012) 22:907–913
DOI 10.1007/s10895-011-1029-6

ORIGINAL PAPER

A Study of the Intrinsic Autofluorescence of Poly (ethylene glycol)-co-($_L$-Lactic acid) Diacrylate

Yu-Chieh Chiu · Eric M. Brey · Víctor H. Pérez-Luna

Received: 31 October 2011 / Accepted: 19 December 2011 / Published online: 5 January 2012
© Springer Science+Business Media, LLC 2012

Abstract Poly (ethylene glycol)-co-($_L$-Lactic acid) diacrylate (PEG-PLLA-DA) copolymers have been extensively investigated for a number of applications in medicine. PEG-PLLA-DA is biodegradable and the human body can process its degradation products. In this study, we describe the autofluorescence of PEG-PLLA-DA copolymers and compared it to the fluorescence of poly(ethylene glycol) diacrylate (PEG-DA) and the precursor molecules used for their synthesis. In addition, we examined the influence of pH on the fluorescence spectra. We found that PEG-PLLA-DA exhibits higher fluorescence than PEG-DA and all reagents involved in the synthesis of PEG-PLLA-DA. The fluorescence of PEG-PLLA-DA was affected by pH with fluorescence decreasing at high pH values. At high pH, PEG-PLLA-DA could not polymerize into hydrogels and exhibited a dramatic decrease in autofluorescence, suggesting that hydrolysis of the ester bond affected its autofluorescence. At low pH, PEG-PLLA-DA exhibited higher fluorescence and it was able to form crosslinked hydrogels. The autofluorescence of PEG-PLLA-DA could be exploited to monitor polymer degradation and material structure without the need to introduce exogenous fluorescent probes. The origin of fluorescence is not clear at this point in time but it appears to result from a synergetic effect of both lactate units and diacrylate groups in the PEG-PLLA-DA backbone. The observed autofluorescence of PEG-PLLA-DA persists after reaction of the acrylate groups in the polymerization reaction. This autofluorescence is advantageous because it could assist in the study of polymers used for drug delivery and tissue engineering applications.

Keywords Autofluorescence · Hydrogel · Poly (ethylene glycol)-co-($_L$-Lactic acid) diacrylate · Poly (ethylene glycol) diacrylate

Y.-C. Chiu · E. M. Brey
Department of Biomedical Engineering,
Illinois Institute of Technology,
Chicago, IL, USA

E. M. Brey
Research Service, Hines Veterans Administration Hospital,
Hines, IL, USA

V. H. Pérez-Luna (✉)
Department of Chemical and Biological Engineering,
Illinois institute of Technology,
10 West 33rd Street,
Chicago, IL 60616, USA
e-mail: perezluna@iit.edu

Introduction

Polyethylene glycol diacrylates (PEG-DA)-based polymers are important hydrogel precursors commonly investigated for tissue engineering, drug delivery and regenerative medicine applications. PEG-DA materials are biocompatible and resistant to protein adsorption [1, 2]. PEG-based materials can be made degradable by hydrolysis by incorporation of poly($_L$-Lactic acid) blocks into the PEG backbone to generate PEG-PLLA-DA copolymers (Fig. 1) [3–5]. This approach allows degradation of polymers that have been investigated for applications in drug delivery[6], bone fixation devices[7], and cartilage repair [8]. Although there are clinical studies on the use of PEG-DA and PEG-PLLA-DA hydrogels as biomaterial scaffolds, there continues to be significant basic research examining the materials underlying properties and performance in biomedical applications. Despite this research, to our knowledge there have been no studies to examine the intrinsic autofluorescence of these materials.

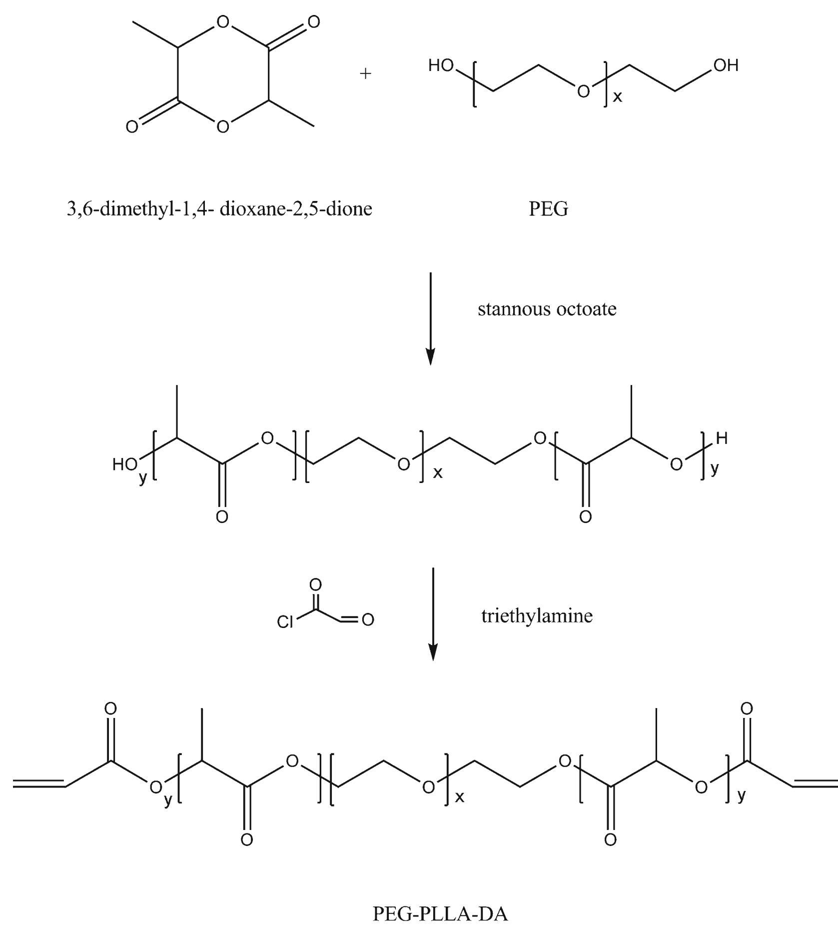

Fig. 1 Schematic representation of the synthesis of PEG-PLLA-DA

The incorporation of fluorescent markers into biomaterials is often used to assist in the study of drug delivery [9], imaging of material structures [10], and measuring degradation rate of biodegradable materials[11]. A common approach is to incorporate fluorescent probes into the materials to monitor release kinetics and degradation [12, 13]. However, the incorporation of these probes is challenging in terms of achieving efficient and extensive labeling of the material, they may alter material properties and often results in some toxicity. A hydrogel possessing intrinsic fluorescence would make these studies more convenient and would eliminate potential artifacts that could arise in the process of introducing fluorescent labels.

In this work, the autofluorescence of PEG-DA, and PEG-PLLA-DA are reported. Interestingly, none of the building blocks of these polymers, including lactic acid (LA), PEG, PEG-PLLA or acrylate (measured as acrylic acid(AA)) exhibited significant fluorescence. The fluorescence of PEG-PLLA-DA is significantly affected by pH. This autofluorescence could allow for the noninvasive monitoring of PEG-PLLA-DA hydrogel structure and degradation without resorting to exogenous fluorescent molecules. The findings on the intrinsic fluorescence of these important biomaterials could prove useful for imaging and measuring the degradation of those hydrogels in tissue engineering applications.

Experimental

Materials and Methods

PEG (Mn≈3400), acryloyl chloride (98%), triethylamine (99.5%), 3,6-dimethyl-1,4- dioxane-2,5-dione, acrylic acid, and 2-hydroxy-2-methylpropiophenone (Irgacure 1173) were obtained from Sigma (St. Louis, MO). Sodium chloride (99.5%), microscopy slides, quartz cell, dichloromethane (99.9%), and ethyl ether (anhydrous) were from Fisher Scientific (Pittsburgh, PA).

Synthesis of PEG-PLLA-DA

Synthesis of PEG-PLLA and PEG-PLLA-DA was based on the procedures originally developed by Hubbell [3] as described by Chiu et al. [14]. All glassware and stir bars were cleaned and dried in a vacuum oven at 120 °C for 24 h prior to use. The following procedure was implemented for a 10-gram synthesis of PEG-PLLA. Ten g of PEG mixed with 2.12 g of 3,6-dimethyl-1,4- dioxane-2,5-dione were lyophilized overnight. The lyophilized PEG, 3,6-dimethyl-1,4- dioxane-2,5-dione and 40 μL of stannous octoate were placed in a round bottom flask. The flask was filled with

argon and subjected to vacuum three times in order to ensure the absence of trace water and oxygen. In order to perform the reaction at a uniform temperature the entire flask was submerged in an oil bath. The temperature of the oil bath was brought to 140 °C and the mixture was allowed to react for 4 h. The resulting products were dissolved in 40 mL of dichloromethane, filtered with glass fiber filter (GF/F, Whatman, Maidstone UK) and precipitated in ice cold ethyl ether three times.

To synthesize PEG-PLLA-DA, 10 g of PEG-PLLA were lyophilized and placed into a three neck round bottom flask with 60 mL of dichloromethane. Two moles of triethylamine per mole of PEG-PLLA were added into the flask and stirred for 5 min under an inert Ar gas atmosphere. Next, four moles of acryloyl chloride per mole of PEG-PLLA were added dropwise and reacted overnight in the dark under Ar gas atmosphere. The resulting products were washed with 5 ml of 2 M K_2CO_3 and then precipitated into 2 L of ice-cold ethyl ether to remove the residual acryloyl chloride. The extent of reaction, structure and purity of the products were verified by Fourier Transform Infrared Spectroscopy (FTIR) (Tensor 27 FTIR; Bruker; Billerica, MA) and proton Nuclear Magnetic Resonance ^{1}H NMR (Advance 300 Hz; Bruker, Billerica, MA). To perform ^{1}H NMR, the products were dissolved in $CDCl_3$ with 0.05% v/v of tetramethylsilane (TMS) added as an internal calibration standard.

Hydrogel Preparation

Unless otherwise noted, all reagents were dissolved in deionized (DI) water obtained from a Millipore system (conductivity was greater than 18 MΩ cm). The hydrogel precursor solution consisted of 11.63 mM PEG-PLLA-DA and was prepared by dissolving 50 mg of PEG-PLLA-DA (Mw≈4300) in 1 mL of DI water. Irgacure 1173 (0.5% w/v) was added to the precursor as the photoinitiator. Two and a half mL of precursor were placed in a quartz cuvette and polymerized under UV light (365 nm) for 5 min. Hydrogels were measured for emission spectra immediately after polymerization. Thin hydrogel slabs for absorbance measurements were prepared between two quartz slides by using microscope coverslips as spacers (thin hydrogel slabs were needed because the hydrogels exhibited strong absorbance). Six hundreds μL of precursor were injected between two quartz slides and polymerized under UV light (365 nm) for 5 min.

Fluorescence Measurements

The absorbance and emission spectra of the different reagents: macromers, hydrogel precursors and hydrogels were performed in quartz cuvettes. A solution of 3,6-dimethyl-1,4- dioxane-2,5-dione was prepared in DI water and stored at room temperature for 3 days to allow complete hydrolysis to lactic acid before measuring the emission of fluorescence. The pH of solutions was adjusted to acidic and basic conditions using 1 N HCl and 1 N NaOH respectively. The fluorescence of samples was measured using a FluoroMax-3 spectrofluorometer (Horiba Ltd. Edison, NJ) using a excitation wavelength of 355 nm. The absorbance of these samples was also measured using a UV-2401 Shimadzu spectrophotometer using DI water as blank.

Monitoring of PEG-PLLA-DA Hydrogel Degradation Though its Intrinsic Fluorescence

Hydrogels for degradation studies were synthesized by polymerizing 200 μL of hydrogel precursor solution in 48 well plates using UV light (365 nm) for 5 min. After polymerization, the hydrogels were incubated in 1 mL PBS and stored in 37 °C. The supernatant solution was removed and replaced with fresh PBS every other day. The gel and removed solution (containing degradation products) were placed in 48 well plates respectively and their fluorescence was determined using a Microplate spectrophotometer (Spectromax, Molecular devices, Sunnyvale, CA) with excitation 355 nm and emission 460 nm. Data were subtracted from a PBS blank control.

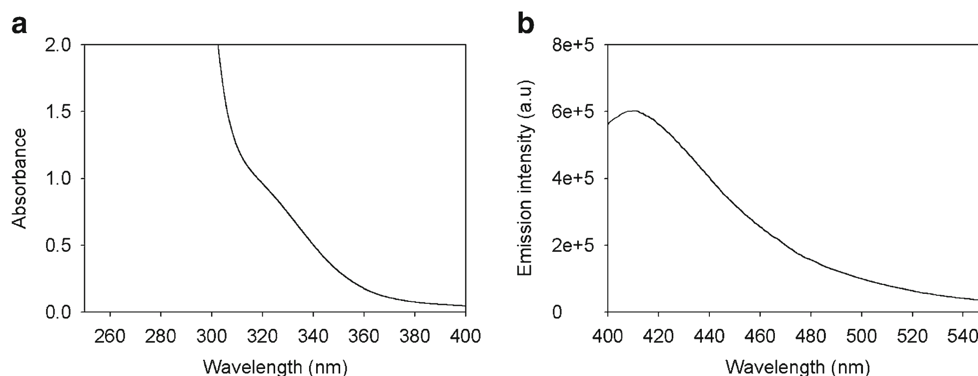

Fig. 2 (**a**) Absorbance spectra, and (**b**) emission spectra of PEG-PLLA-DA hydrogels

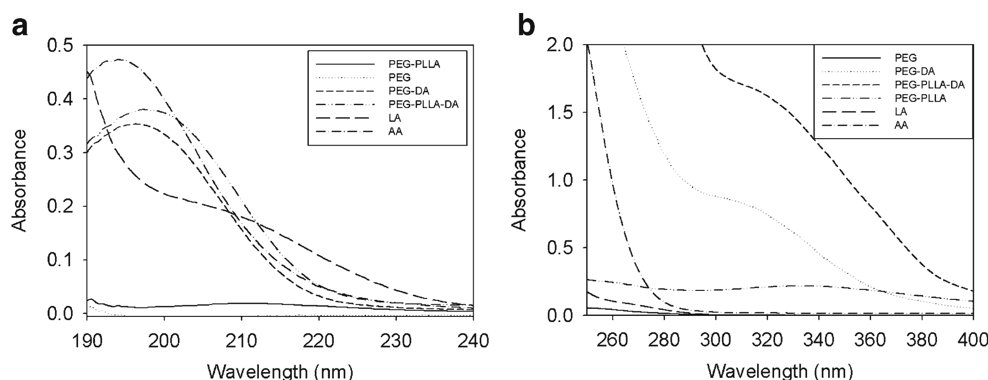

Fig. 3 **a** Absorption spectra of 11.6 nM PEG-PLLA, PEG, PEG-DA, PEG-PLLA-DA, and LA respectively. (Except 23.2 nM AA); **b** Absorption spectra of 11.6 mM PEG-PLLA, PEG, PEG-DA, PEG-PLLA-DA, and LA respectively. (Except 23.2 mM AA)

Generating Porous PEG-PLLA-DA Hydrogel

Porous PEG-PLLA-DA hydrogels were prepared using a salt leaching procedure. A 58.15 mM solution of PEG-PLL-DA polymer precursor was prepared using 250 mg of PEG-PLLA-DA polymer dissolved in 1 mL of dichloromethane. Irgacure 1173 was added as a photoinitiator (5% (w/v)). Two hundreds and 50 mg of sieved salt (150-100 μm) and 250 mL of precursor were placed in a 1.5 ml centrifuge tube. The tube was vortexed for 45 s and placed upside down allowing the salt to settle in to the cap for 1 min. A microscope slide was used to cover the solution, carefully avoiding bubble formation. The solution was then polymerized by irradiation under UV for 10 min. The sample was then rotated and polymerized for an additional 10 min. The microscope slide was removed and the dichloromethane allowed to evaporate overnight. The resulting gels were placed in a 50 mL sterile centrifuge tube with 20 ml DI water containing 4 mg/mL of gentamicin sulfate (to prevent microbial growth), and then immediately exposed to a vacuum (0.035 mBar) for 15 min to remove air trapped in the porous gels. The water was changed 4 times for 1 day to completely leach out the salt crystals. In the salt leaching process, the degree of hydrolysis of the porous hydrogel was considered to be very minor and did not affect fluorescent intensity of the porous hydrogel.

Confocal Imaging of Pore Structure

The porous PEG-PLLA-DA hydrogel was imaged using a PASCAL laser scanning microscopy system from Carl Zeiss MicroImaging, Inc. (Thornwood, NY). A 488 nm laser was used as the excitation source and a 505 nm low pass filter was used for the emission of fluorescence. Images had x and y resolution of 3.5 μm/pixel and z resolution of 20 μm/pixel. The serial confocal images were exported into Axiovision 4.5 (Carl Zeiss, Göttingen, Germany) for reconstruction into 3-D images.

Results and Discussion

To the best of our knowledge, the intrinsic fluorescence of PEG-PLLA-DA polymers has not been reported previously. The absorbance spectra of a hydrogel prepared using 11.36 mM PEG-PLLA-DA shows a broad shoulder peak from 310 nm to 360 nm (Fig. 2a). The fluorescent emission spectrum of this hydrogel shows a broad peak over a long range of wavelengths with the peak maximum occurring around 410 nm. The characteristics of this broad emission peak indicate that the observed spectrum is not due to scattering from the sample but most likely is due to fluorescence (Fig. 2b). By creating hydrogels within a quartz cuvette and between quartz slides without interfaces due to

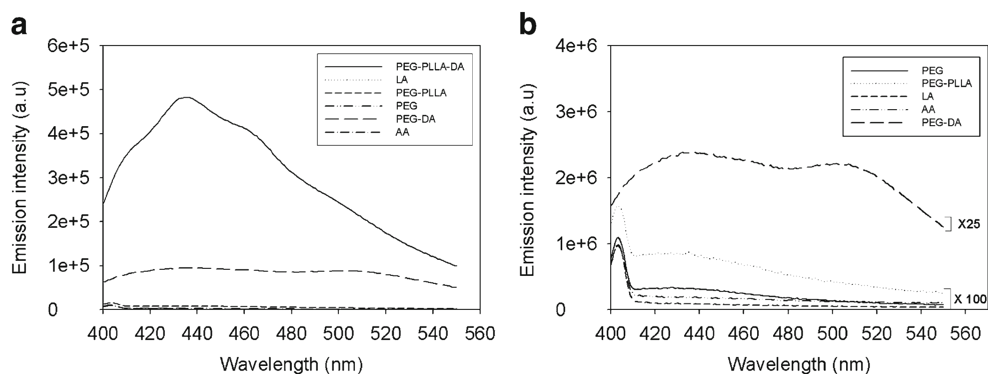

Fig. 4 **a** Emission spectra of 11.6 mM PEG-PLLA-DA, LA, PEG-PLLA, PEG, and PEG-DA respectively.(except 23.3 mM AA). **b** Rescaled emission spectra of Fig. 3a without showing emission of PEG-PLLA-DA

shrinkage or bubbles, it was possible to minimize scattering in these measurements. These measurements of absorbance and emission spectra indicate that there is autofluorescence of PEG-PLLA-DA hydrogels (Fig. 2).

In order to investigate the origin of the autofluorescence of PEG-PLA-DA hydrogel, all reagents used in the synthesis of PEG-PLLA-DA hydrogel (PEG, PEG-PLLA, LA, and AA) were characterized by optical absorption spectroscopy. Both PEG-DA and PEG-PLLA-DA were characterized by optical absorption spectroscopy as well. All solutions used for these measurements had identical molar concentrations (11.6 mM) except AA, which had twice the molar concentration of the others due to the presence of two moles of acrylate per mole of PEG-PLLA-DA. The absorption spectra of diluted solutions of PEG or PEG-PLLA, 11.6 nM, did not show significant absorbance peaks from 190 nm to 240 nm wavelength range (Fig. 3a). However, PEG-DA, PEG-PLLA-DA, lactate, and acrylic acid, all showed significant absorbance in the 190-240 nm wavelength range. At this low concentration (11.6 nM) there was no significant light absorption above 240 nm for any of these reagents. The absorbance spectra of more concentrated solutions, 11.6 mM, shows that both PEG-DA and PEG-PLLA-DA exhibit strong light absorption in the 300–370 nm wavelength range while the molecules used for their synthesis show no absorbance in this region (Fig. 3b). The presence of those absorbance peaks support the hypothesis that autofluorescence of PEG-DA and PEG-PLLA-DA is observed when these materials are excited using 355 nm light. However, the fact that the precursor molecules for synthesis of PEG-DA and PEG-PLLA-DA do not exhibit such strong absorbance nor fluorescence appears to indicate that there is a synergistic effect of these molecules that gives rise to the fluorescence of PEG-DA and PEG-PLLA-DA.

The emission spectra indicates that PEG-PLLA-DA has 25 times stronger fluorescence than PEG-DA (Fig. 4a) and that PEG-PLLA-DA has 100 times stronger emission intensity than acrylic acid, lactic acid, PEG or PEG-PLLA (Fig. 4b). Although the acrylation of either PEG or PEG-PLLA significantly increases autofluorescence, this increase is substantially larger with PEG-PLLA. This indicates that the there is a synergetic effect for increasing the autofluorescence of PEG- PLLA-DA or PEG-DA when attaching acrylate groups to PEG-PLLA backbone or PEG.

In order to further investigate the auto fluorescence of PEG-PLLA-DA, the effect of pH on fluorescence was investigated. It was found that the fluorescence of PEG-PLLA-DA solution decreased when the pH was sequentially increased from a value of pH=5 to pH=9, 11, 12 and 13 (Fig. 5a). The decrease of fluorescence of PEG-PLLA-DA is likely due to hydrolysis of ester bonds with the concomitant loss of acrylic groups. This would indicate a role of the acrylate groups conjugated to PEG or PEG-PLLA in the

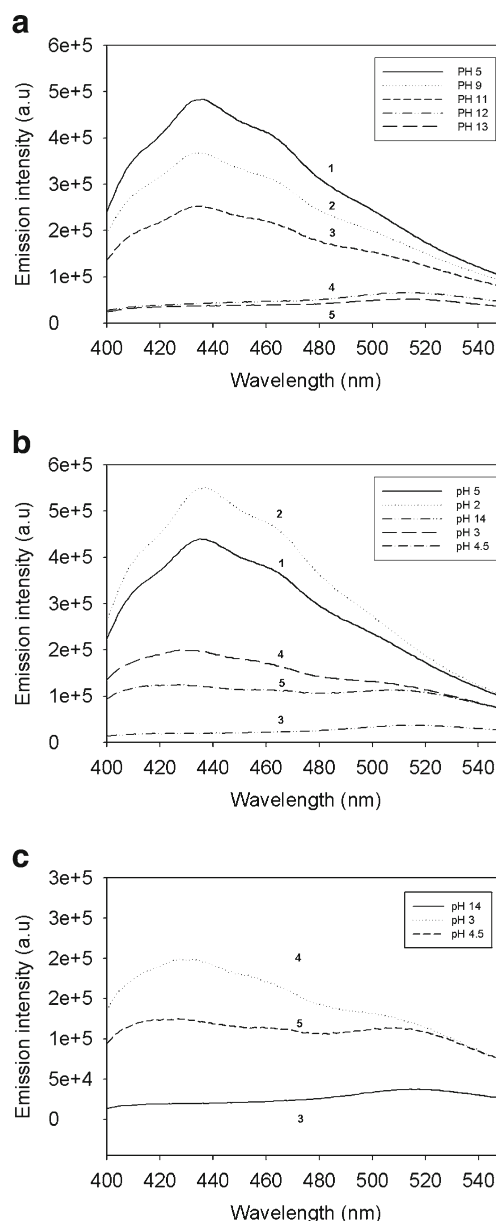

Fig. 5 **a** Emission spectra of increasing the pH of 11.63 mM PEG-PLLA-DA hydrogel precursor; **b** Emission spectra of varying pH of 11.63 mM PEG-PLLA-DA hydrogel precursor from first pH5 to 2, 14, 3 and 4.5; **c** Rescaled emission spectra of Fig. 5b. (numbers in the spectra indicate the sequence in which the pH was changed i)

observed fluorescence. To further investigate this process further, the pH was first decreased from pH 5 to pH 2, which resulted in a slight increase in fluorescence of the hydrogel precursor solution (Fig. 5b). If initiator was added to this solution it was able to polymerize and form a hydrogel, indicating that the acrylate groups had not been hydrolyzed. If the pH was instead increased from 2 to 14 the resulting solution was not able to polymerize after adding initiator. This indicated loss of the acrylate groups due to hydrolysis of PLLA. In a third experiment, the pH of the PEG-PLLA-

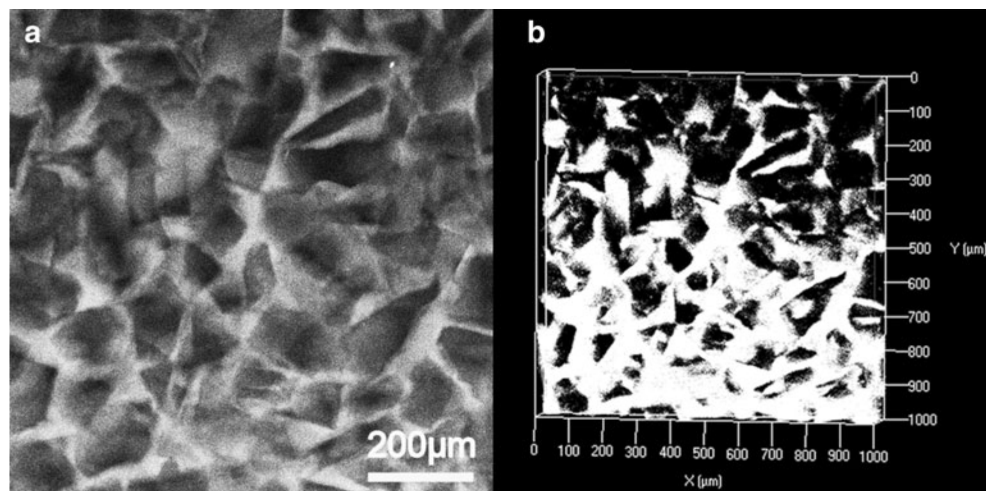

Fig. 6 (**a**) 2 D projection and (**b**) 3 D volume renderings of a confocal image of porous PEG-PLLA-DA by salt leaching

DA solution was brought back from 14 to 4.5; and the resulting solution was not able to polymerize after adding initiator (Fig. 5b and c), indicating that the pH effect, by its nature of being irreversible when increasing the pH was due to hydrolysis. These experimental observations support the hypothesis that hydrolysis of PEG-PLLA-DA resulted in its loss of fluorescence. It was also observed that the fluorescence of PEG-PLLA-DA decreased when the temperature increased (not shown). These observed changes in the emission spectra further lend support to the hypothesis that the images obtained under the microscope and the measurements obtained in the spectrofluorometer are autofluorescence and not a light scattering phenomenon.

Given that PEG-PLLA-DA hydrogels exhibit autofluorescence we wanted to examine how this can be used to study degradation and structure of the materials. The ability to dynamically image and monitor degradable porous scaffold is especially useful for biomaterials used in tissue engineering and drug delivery. Although 3-D reconstructed images can be acquired by selective partitioning of proteins into the pores of PEG hydrogel [15], the methods to nondestructively image the architecture of degrading biomaterials under fully swelled conditions are limited. Given the autofluorescence of PEG-PLLA-DA hydrogels, it is possible to study the architecture of porous scaffolds and degradation of this material without the need to incorporate fluorescent labels. Confocal microscopy revealed that the autofluorescence could be used to provide 2-D (Fig. 6a) and 3-D (Fig. 6b) images of the porous structures of salt leached PEG-PLLA-DA hydrogel scaffolds. This observation is promising and could provide a platform to monitor the architecture of degrading of PEG-PLLA-DA scaffold in vitro and possibly in vivo for tissue engineering applications.

In the past, measuring the degradation of PEG-PLLA-DA hydrogels required either assessing multiple hydrogels samples at desired time points by mechanical or swelling properties or incorporating fluorescent probes [16]. In this study, the degradation of a PEG-PLLA-DA hydrogel was monitored directly by measuring autofluorescence of the hydrogels or fluorescence present in the surrounding solution due to degradation products in vitro (Fig. 7). This was performed without disturbing the hydrogels or incorporating fluorescent probes. This study illustrates how the autofluorescence of PEG-PLLA-DA becomes advantageous for monitoring in vitro degradation of PEG-PLLA-DA hydrogels which are under investigation for tissue engineering applications.

Conclusions

The autofluorescence of PEG-PLLA-DA solutions, polymerized hydrogels, and degradation products has been studied here. The origin of the fluorescence of PEG-PLLA-DA hydrogel appears to originate for PEG-PLLA with end caped acrylate groups. This observation is supported by the fact that the molecules used to synthesize PEG-PLLA-DA or PEG-DA do not exhibit fluorescence by themselves and that

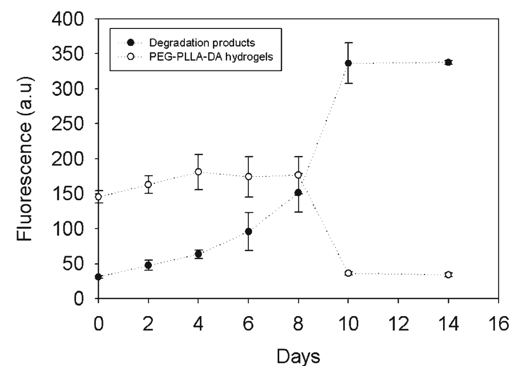

Fig. 7 PEG-PLLA-DA hydrogel degradation monitoring by hydrogel and degradation products (cumulative fluorescence of degradation products)

hydrolysis caused by increases in pH resulted in irreversible loss of fluorescence. Both temperature and pH affect the fluorescence of PEG-PLLA-DA, which indicates that the phenomenon observed is due to auto fluorescence rather than light scattering. The intrinsic autofluorescence of this material can be used to image porous scaffolds in 3-D and also to monitor their degradation. The autofluorescence of this material allow studying these systems in situ, thus permitting to obtain information useful in imaging, drug delivery and regenerative medicine.

Acknowledgments This research was supported by funding from Veterans Administration.

References

1. Graham NB, Zulfiqar M, Nwachuku NE, Rashid A (1990) Interaction of poly(Ethylene Oxide) with solvents.4. Interaction of water with poly(Ethylene Oxide) cross-linked hydrogels. Polymer 31(5):909–916
2. Chiu YC, Cheng MH, Engel H, Kao SW, Larson JC, Gupta S, Brey EM (2011) The role of pore size on vascularization and tissue remodeling in PEG hydrogel. Biomaterials
3. Sawhney AS, Pathak CP, Hubbell JA (1993) Bioerodible hydrogels based on photopolymerized poly(ethylene glycol)-co-poly(a-hydroxy acid) diacrylate macromers. Macromolecules 26 (4):581–587
4. Sawhney AS, Hubbell JA (1990) Rapidly degraded terpolymers of dl-lactide, glycolide, and e-caprolactone with increased hydrophilicity by copolymerization with polyethers. J Biomed Mater Res 24 (10):1397–1411
5. Han DK, Hubbell JA (1997) Synthesis of polymer network scaffolds from L-Lactide and poly(ethylene glycol) and their interaction with cells. Macromolecules 30(20):6077–6083
6. Peppas NA, Bures P, Leobandung W, Ichikawa H (2000) Hydrogels in pharmaceutical formulations. Eur J Pharm Biopharm 50 (1):27–46
7. Hattori K, Tomita N, Yoshikawa T, Takakura Y (2001) Prospects for bone fixation - development of new cerclage fixation techniques. Mat Sci Eng C-Bio S 17(1–2):27–32
8. Chu CR, Coutts RD, Yoshioka M, Harwood FL, Monosov AZ, Amiel D (1995) Articular cartilage repair using allogeneic perichondrocyteseeded biodegradable porous polylactic acid (PLA): a tissue-engineering study. J Biomed Mater Res 29(9):1147–1154. doi:10.1002/jbm.820290915
9. Feng SS, Ruan G, Li QT (2004) Fabrication and characterizations of a novel drug delivery device liposomes-in-microsphere (LIM). Biomaterials 25(21):5181–5189. doi:10.1016/J.Biomaterials.2003.12.013
10. Rounds RM, Ibey BL, Beier HT, Pishko MV, Cote GL (2007) Microporated PEG spheres for fluorescent analyte detection. J Fluoresc 17(1):57–63. doi:10.1007/S10895-006-0143-3
11. Kloxin AM, Kloxin CJ, Bowman CN, Anseth KS (2010) Mechanical properties of cellularly responsive hydrogels and their experimental determination. Adv Mater 22(31):3484–3494. doi:10.1002/Adma.200904179
12. Nagahama K, Fujiura K, Enami S, Ouchi T, Ohya Y (2008) Irreversible temperature-responsive formation of high-strength hydrogel from an enantiomeric mixture of starburst triblock copolymers consisting of 8-arm PEG and PLLA or PDLA. J Polym Sci Pol Chem 46(18):6317–6332. doi:10.1002/Pola.22943
13. Vermonden T, Jena SS, Barriet D, Censi R, van der Gucht J, Hennink WE, Siegel RA (2010) Macromolecular diffusion in self-assembling biodegradable thermosensitive hydrogels. Macromolecules 43(2):782–789. doi:10.1021/Ma902186e
14. Chiu YC, Larson JC, Perez-Luna VH, Brey EA (2009) Formation of microchannels in Poly(ethylene glycol) hydrogels by selective degradation of patterned microstructures. Chem Mater 21 (8):1677–1682. doi:10.1021/Cm803520j
15. Chiu YC, Larson JC, Isom A, Brey EM (2010) Generation of porous poly(Ethylene Glycol) hydrogels by salt leaching. Tissue Eng Part C-Me 16(5):905–912. doi:10.1089/Ten.Tec.2009.0646
16. Metters AT, Bowman CN, Anseth KS (2000) A statistical kinetic model for the bulk degradation of PLA-b-PEG-b-PLA hydrogel networks. J Phys Chem B 104(30):7043–7049. doi:10.1021/jp000523t