

Size optimization of biodegradable fluorescent nanogels for cell imaging

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Submitted 2010, Accepted September 2011, Published November 2011.

Abstract: Photocrosslinked biodegradable photoluminescent polymers (PBPLPs) nanogels have been engineered by free-radical crosslinking of a vinyl-containing fluorescent prepolymer. The free radical crosslinking was studied by varying the concentrations of prepolymer, crosslinker, and photoinitiator. We observed that the polymer concentration showed inconsistent influence on nanogel particle sizes when it ranged from 0.3 to 0.8 %(w/v). However, the concentrations of crosslinker (acrylic acid) and photoinitiator (2,2'-azobis(2-methylpropionamide) dihydrochloride) influenced the average diameter of PBPLP nanogels. Increasing the concentration of crosslinker resulted in an increase in the average diameter of nanogels, while an increase in the concentration of photoinitiator resulted in a decrease in the average diameter of PBPLP nanogels. These nanogels were spherical in morphology as confirmed by transmission electron microscopy. When incubated with NIH 3T3 fibroblast cells, PBPLP nanogel particles were uptaken within the cytoplasm, resulting in a strong fluorescent signal observed under fluorescence microscopy. With the potential to act as both a vehicle for drug delivery and imaging probes, the development of PBPLP nanogels represent a new direction in developing nanobiomaterials in theranostic nanomedicine.

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1. Introduction:

Hydrogels are crosslinked networks of hydrophilic polymers.¹ They have high water retention abilities and a rubbery nature that is comparable to natural tissue.² Their applications can be found in numerous biomedical and pharmaceutical settings ranging from contact lenses to scaffolds for tissue regeneration.^{3,4} More recently, hydrogels have also been fabricated into microstructures and nanostructures for cell delivery and drug delivery.^{5,6} Nanogels are useful in drug delivery due to their small size, which allows for easier cellular uptake as well as controlled properties and drug release.⁷ For example, Poly(*N*-isopropylacrylamide) one of the most extensively studied polymers, which is crosslinked through free radical crosslinking, has been used for ~~in~~ controlled drug delivery in the forms of nano-/microgels.⁸ In addition, when a fluorescent probe is encapsulated or conjugated with nanogel networks, they can be used as imaging probes for bioimaging applications, such as early cancer detection.^{9,10}

In our previous work, we reported the discovery of the first biodegradable photoluminescent polymers (BPLPs). BPLP demonstrated bright fluorescence with high quantum yields (up to 79%) and tunable fluorescence emission (up to 825 nm).¹¹ These biodegradable fluorescent polymers have tremendous potential in the field of bioimaging and drug delivery. However, these BPLPs may not be suitable for the fabrication of stable nanoparticles due to their low molecular weight.

In the present work, we have synthesized another class of polymers for BPLP family that can be fabricated into stable nanogels via free radical

crosslinking referred to as photocrosslinked biodegradable photoluminescent polymers (PBPLPs). Significant efforts are placed on the fabrication of nanogels using PBPLPs. These nanogels are fabricated by crosslinking vinyl-containing PBPLP prepolymers through a free radical polymerization in the presence of crosslinker. We hypothesize that the concentrations of prepolymer, crosslinker, and initiator have a significant effect on the average size and size distribution of these crosslinked photoluminescent nanogels. Size is important when fabricating nanogels for biomedical applications because the size of nanoparticles affects biodegradability, cellular uptake, and drug release.⁹ We determine the ideal composition for the fabrication of PBPLP nanogels in this work.

2. Experimental Methods:

All chemicals, cell culture medium, and supplements were purchased from Sigma-Aldrich (St. Louis, MO), except where mentioned otherwise. All chemicals were used as received.

2.1. Synthesis of PBPLP

PBPLP prepolymer was synthesized according to previously published methods with minor modifications.¹¹ Briefly, 0.05 moles of citric acid, 0.1 moles of polyethylene glycol ($M_w = 200$ Da), and 0.05 moles of maleic acid were melted in a 250 mL three necked round bottom flask fitted with an inlet adapter and outlet adapter by stirring the contents in the flask at a temperature of 160 °C under nitrogen gas flow for 20 minutes. Once the constituents melted, 0.01 moles of L-cysteine were added to the reaction flask. Following this, the temperature was reduced to 140 °C and

the pressure was reduced to 50 mTorr. The reaction was continued until the desired viscosity was observed. The prepared prepolymers were dissolved in deionized water and dialyzed with a 500 Dalton molecular weight cut off membrane for 2 days followed by lyophilization to obtain a purified prepolymer of PBPLP.

2.2. Fabrication of BPLP nanogels

0.25 g of PBPLP prepolymer was deposited into a 250 mL beaker, and 50 ml of the deionized (DI) water was then added to it. The PBPLP prepolymer was dissolved completely in water forming a homogeneous solution. Then, using a pipette, 50 μ L of crosslinker (acrylic acid) was added to the solution followed by 0.01 g of the photoinitiator (PI) (2,2'-azobis(2-methylpropionamide) dihydrochloride).

The beaker containing the above solution was then placed into a sonicator, and was sonicated for one cycle lasting about 15 minutes. After the first round of sonication, a UVP 365 nm long-wave (ca. 4 mW/cm², model 100AP, Blak-Ray) was placed over the beaker which was still kept in the sonicator, and one more round of sonication was performed while the UV light initiated the crosslinking reaction of the prepolymer. Crosslinking was indicated by the solution becoming cloudy after these two rounds of sonication.

The solution was then poured equally into two ultracentrifuge tubes, using a scale to ensure that each tube contained equal amounts. After placing the tubes into opposite slots in the ultracentrifuge machine, the ultracentrifuge was allowed to run at 25,000 rpm for 30 minutes at room temperature. After the ultracentrifuge completed its cycle, two nanoparticle pellets were visible at the

base of each tube. The pellets were resuspended at a concentration of 1% (w/v) in fresh DI water for further characterization.

Various concentrations of prepolymer (0.3, 0.4, 0.5, and 0.8) % (w/v), crosslinker (0.05, 0.1, and 0.2)% (v/v), and PI (0.01, 0.02, and 0.04)% (w/v) were used to fabricate PBPLP nanogels to understand their effect on the average size and size distribution.

2.3. Size measurement of PBPLP nanogels.

A cuvette was filled with approximately 1/4 nanoparticles solution and 3/4 fresh DI water and then placed into a dynamic light scattering (DLS) machine in order to record the average particle size, polydispersity, and standard deviation. Furthermore, TEM 2 JEOL 1200EX II was used to visualize PBPLP nanogels to observe the morphology of these nanogels.

2.4. Nanogel cellular uptake study

NIH 3T3 fibroblasts were used as model cells for cellular uptake evaluation. The cells were cultured in 25 cm² tissue culture flasks with Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The culture flasks were kept in an incubator maintained at 37 °C, 5% CO₂ and 95% humidity. The cells were further trypsinized, centrifuged and suspended in the culture medium to obtain a seeding density of 1×10⁵ cell/ml. 1 ml of the cell suspension was added on top of a glass slide in the culture dish. After 24 h of incubation at 37 °C, the medium was replaced with 1 ml of PBPLP nanogels in culture medium. After 24 h of incubation of nanogels with the cells, fluorescent images of the cells were taken under a fluorescence microscope with a 4',6-diamidino-2-phenylindole

(DAPI) filter to demonstrate the cellular uptake of these nanogels.

3. Results and Discussion Section:

3.1. PBPLP prepolymer synthesis

Water soluble biodegradable photocrosslinkable fluorescent prepolymer was synthesized through a polycondensation reaction. Citric acid was used as multifunctional monomer¹² that was linearly connected to poly ethylene glycol by biodegradable ester bonds. Similarly, maleic acid was used as a vinyl-containing monomer that was linked to polyethylene glycol by ester bonds to introduce photocrosslinkability.¹³ Finally, L-cysteine was reacted to this linear prepolymer chain to form a fluorescent 6-membered ring as described previously.¹¹ Detailed characterization of the chemical and fluorescent properties of this polymer will be discussed in a future publication. Herein we will focus on the fabrication and characterization of nanogels of PBPLP.

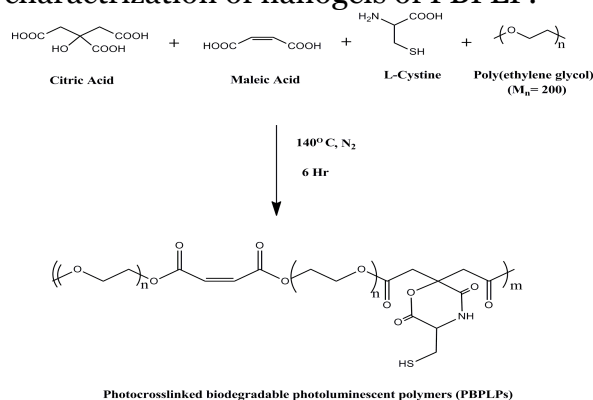


Figure 1. Schematic representation of PBPLP synthesis

3.2. Fabrication of PBPLP nanogels

An ultra violet (UV) light induced free radical crosslinking system was employed to fabricate PBPLP nanogels in this experiment. PBPLP prepolymer was mixed with terminal alkene crosslinker and a photoinitiator. The

system was agitated by a low power sonicator while crosslinking the prepolymer into nanogels. It was realized that the concentrations of prepolymer, crosslinker, and photoinitiator have a significant effect on the overall crosslinking kinetics of the polymer. Since the average size distribution of these nanoparticles is highly governed by the crosslinking kinetics of the reaction, we investigated the effects of the concentrations of these parameters on the size and size distribution of nanogels.

3.3. Size and size distribution

The DLS measurements showed no discernable pattern when comparing the nanogel sizes crosslinked by using various prepolymer concentration (Figure 1). Although 0.5 % (w/v) of PBPLP resulted in the smallest nanoparticle size, 437.5 ± 14 nm with polydispersity (PD) of 0.35, the lack of a consistent change versus prepolymer concentration caused this information to be uncertain.

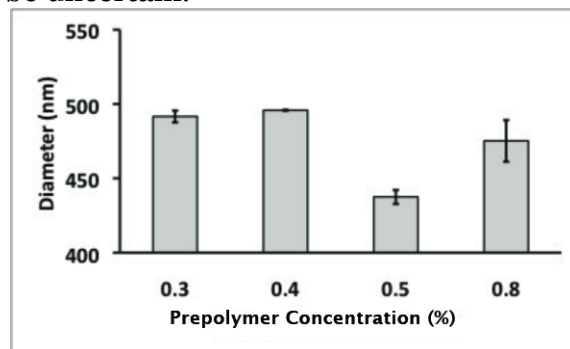


Figure 2. The influence of PBPLP prepolymer concentrations on the average sizes of nanoparticles. At each concentration, crosslinker was constant at 0.1% and PI was constant at 0.02%.

When studying the influence of the amount of crosslinker on nanoparticle sizes, however, a clear pattern was visible (Figure 2). Increasing the amount of crosslinker resulted in larger

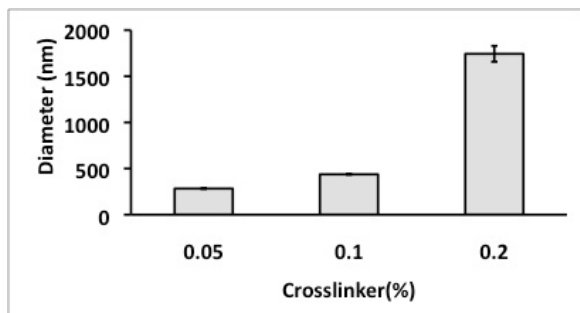


Figure 3. The influence of the amount of crosslinkers on the average sizes of nanoparticle. At each concentration, the amount of PBPLP was constant at 0.5 %, and PI was constant at 0.02%.

nanoparticles size. In this experiment, 0.05% (v/v) of crosslinker resulted in the smallest nanoparticles which are about 283.3 ± 4.6 nm in diameter with PD 0.35. 0.2% (v/v) of crosslinker resulted in micro-sized particles with an average diameter of 1743.2 ± 85.8 nm and PD of 0.25.

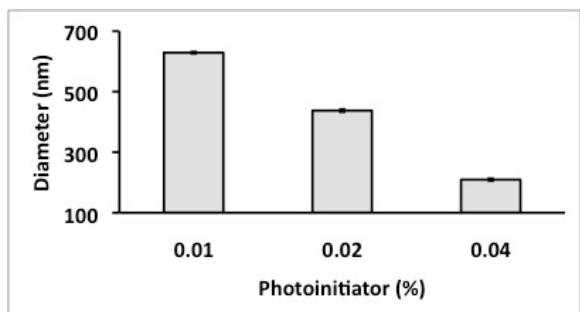


Figure 4. The influence of the amount of PI on the average sizes of nanoparticle . At each concentration, the amount of PBPLP was constant at 0.5 % and the amount of crosslinker was constant at 0.1%.

As shown in figure 3, the average size of PBPLP nanogels was also greatly influenced by the concentration of PI. Increasing the amount of PI resulted in decreased nanoparticles sizes. For example, 0.04% of PI resulted in the smallest size (diameter= 209.367 ± 4 nm, PD=0.32) as compared to those using 0.02% PI (diameter= 437.5 ± 4.7 nm, PD=0.23) and 0.01% (diameter= 628.8 ± 3.1 nm, PD=0.16).

However, no crosslinking was observed in this system with 0.5% of polymer and 0.1% of crosslinker concentration initiated by photoinitiator below the concentration of 0.01%. It may be inferred that the greater amount of PI allows smaller nanoparticles to form because less polymers were crosslinked together when there were more active species (radicals).

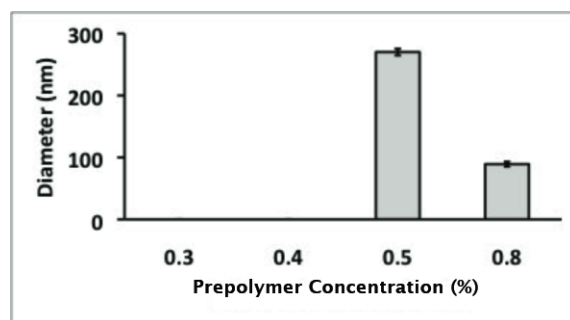


Figure 5. The influence of PBPLP prepolymer concentrations on the average sizes of nanoparticle. In these cases, crosslinker was constant at 0.05% and PI was constant at 0.04%.

Using inferences obtained from Figures 2 and 3, the ideal equation for minimizing nanoparticle size would contain 0.05% (v/v) of crosslinker and 0.04% (w/v) of PI. Because of the inconclusive results obtained from Figure 1, no conclusion could initially be made about the amount of prepolymers that would minimize particle size. Therefore, further experimentation was needed. We kept 0.05% of crosslinker and 0.04 % of PI as constants; the amount of polymers was varied as displayed in Figure 4. Neither 0.3% nor 0.4% of polymer resulted in crosslinked nanoparticles. However, 0.5% and 0.8% of polymer did produce significant amount of nanoparticles. 0.8% of PBPLP resulted in the smallest nanoparticles, 89.2 ± 3.9 nm in diameter and PD of 3.1. Lower concentrations of PBPLP than 0.5% were not enough to allow for crosslinking, perhaps due to a

shortage of PBPLP molecules to pair with the PI. Thus, the larger amount of PBPLP (0.8%) did crosslink and produced the smallest particles.

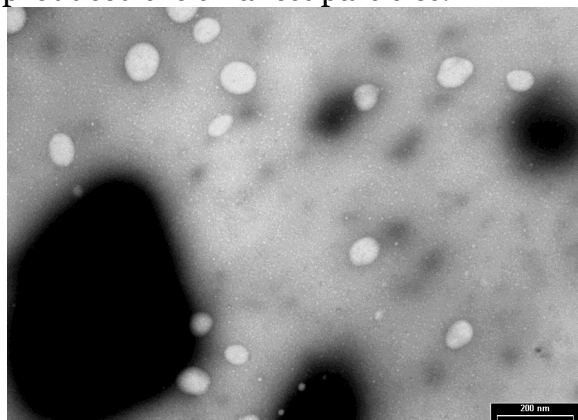


Figure 6. Transmission electron microscopy image of PBPLP nanogels crosslinked with 0.05% (v/v) crosslinker and 0.04% (w/v) PI under UV light for 10 min. The average particle size is 81 ± 16 nm in diameter. Scale bar 200 nm.

To further evaluate the general morphology of PBPLP nanoparticles, PBPLP nanoparticles fabricated with 0.8% of polymer, 0.05% of crosslinker, and 0.04% of PI was visualized under a Transmission electron microscopy (TEM). PBPLP nanogels are circular in morphology with an average diameter of 81 ± 16 nm as shown in Figure 5.

3.4. Nanogel cellular uptake

Cellular uptake of PBPLP nanogels was evaluated against NIH-3T3 cell lines for their potential applications in cellular bioimaging and drug delivery. After incubation of PBPLP nanogels with 3T3 fibroblast cells for 24 hours, cells were easily visible under a fluorescent microscope with a (DAPI) filter. Healthy morphology of the cells was observed as seen in figure 6, indicating that the internalization of PBPLP nanoparticles might not affect the viability and morphology of these cells. PBPLP nanogels were considered to be localized within the cytoplasm of the

cells.

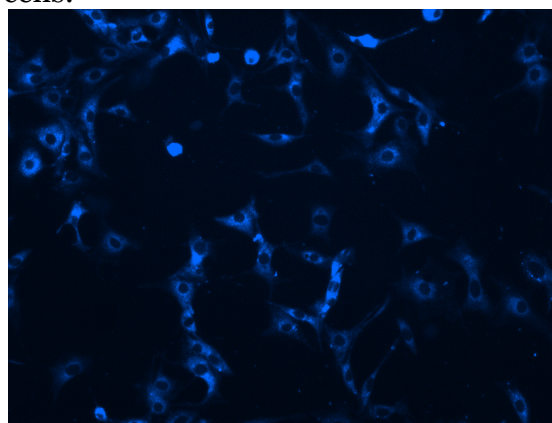


Figure 7: Cellular uptake of PBLPP nanogels in NIH 3T3 fibroblast cells observed under fluorescent microscopy.

Conclusion:

We have synthesized and fabricated the first photocrosslinked biodegradable photoluminescent polymer (PBPLP) nanogels. We evaluated the influence of concentrations of polymer, crosslinker, and PI on the average diameter of these nanogels. Increasing the concentration of PI and decreasing the concentration of crosslinker resulted in a significant decrease in the average size of PBPLP nanogels, whereas polymer concentration showed an inconsistent trend. A formula consisting of 0.8% polymer, 0.05% crosslinker, and 0.04% PI concentration resulted in nanoparticles smaller than 100 nm as confirmed by DLS measurement and TEM. These nanogels were easily uptaken by 3T3 fibroblast cells. As similar to quantum dots, PBPLP nanoparticles can label cells with fluorescent colors. These exciting results demonstrated that PBPLP nanogel particles may potentially be used as drug delivery and imaging probes without using additional photobleaching organic dyes or cytotoxic quantum dots.

Acknowledgements:

I would like to thank Dr. Jian Yang for providing me with the invaluable opportunity to work in his laboratory. The experiences gained from working with graduate students in a laboratory have undoubtedly benefited me. I would also like to express my deep appreciation for Dipendra Gyawali for taking time out of his busy schedule to supervise me as I worked on my project. This work was partially supported by a R21 award EBO09795 and R01 award EBO12575 from National Institute of Biomedical Imaging and Bioengineering (NIBIB), a High Impact High Risk Award RP110412 from Cancer Prevention & Research Institute of Texas (CPRIT), and a National Science Foundation (NSF) CAREER award 0954109.

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