Facile Dye-Initiated Polymerization of Lactide−Glycolide Generates Highly Fluorescent Poly(lactic-co-glycolic Acid) for Enhanced Characterization of Cellular Delivery

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ABSTRACT: Poly(lactic-co-glycolic acid) (PLGA) is a versatile synthetic copolymer that is widely used in pharmaceutical applications. This is because it is well-tolerated in the body, and copolymers of varying physicochemical properties are readily available via ring-opening polymerization. However, native PLGA polymers are hard to track as drug delivery carriers when delivered via subcellular spaces, due to the absence of an easily accessible “handle” for fluorescent labeling. Here we show a one-step, scalable, solvent-free, synthetic route to fluorescent blue (2-aminoanthracene), green (5-aminofluorescein), and red (rhodamine-6G) PLGA, in which every polymer chain in the sample is fluorescently labeled. The utility of initiator-labeled PLGA was demonstrated through the preparation of nanoparticles, capable of therapeutic subcellular delivery to T-helper-precursor-1 (THP-1) macrophages, a model cell line for determining in vitro biocompatibility and particle uptake. Super resolution confocal fluorescence microscopy imaging showed that dye-initiated PLGA nanoparticles were internalized to punctate regions and retained bright fluorescence over at least 24 h. In comparison, PLGA nanoparticles with 5-aminofluorescein introduced by conventional nanoprecipitation/encapsulation showed diffuse and much lower fluorescence intensity in the same cells and over the same time periods. The utility of this approach for in vitro drug delivery experiments was demonstrated through the concurrent imaging of the fluorescent drug doxorubicin (λem = 480 nm, λex = 590 nm) with carrier 5-aminofluorescein PLGA, also in THP-1 cells, in which the intracellular locations of the drug and the polymer could be clearly visualized. Finally, the dye-labeled particles were evaluated in an in vivo model, via delivery to the nematode Caenorhabditis elegans, with bright fluorescence again apparent in the internal tract after 3 h. The results presented in this manuscript highlight the ease of synthesis of highly fluorescent PLGA, which could be used to augment tracking of future therapeutics and accelerate in vitro and in vivo characterization of delivery systems prior to clinical translation.

Poly(lactic-co-glycolic acid) (PLGA) is a synthetic biocompatible copolymer widely used for biomedical applications.1−4 PLGA particulate carriers have been used to deliver therapeutics ranging from low molecular weight drugs, peptides, and proteins to nucleic acids, vaccines, and cells.1,5−10 In addition, PLGA-based scaffolds based on colloidal particles, hydrogels, and fibers have been extensively explored for regenerative medicine.11−15 For therapeutic applications, it is essential to monitor the kinetics of tissue and cellular drug uptake, specific organ accumulation after systemic administration, in vivo biodistribution, and intracellular fate of these therapeutics.14 For in vitro studies, the most common strategy used is to load PLGA nanoparticles with fluorophores, which can then be imaged with wide-field or confocal fluorescence microscopy to determine polymer biodistribution.15 However, the major drawback associated with this approach has been the separation and leakage of the fluorophore from the PLGA nanoparticles, which can result in measurement and experimental artifacts. This is because it is extremely challenging to distinguish between the signal from the loaded nanoparticles and unbound leaking fluorophores,16 which can lead to an overestimation of the nanoparticle uptake.1 Furthermore, free fluorophores may interact with biological systems and can

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induce unwanted biochemical processes, while burst release of dyes from nanoparticles can result in transient high local concentrations and potential toxicity. To enhance fluorescence imaging of PLGA nanoparticle biodistribution and to overcome the challenges associated with fluorophore leaching, PLGA can be covalently linked to fluorophores. However, PLGA itself has a limited number of easily modifiable functional groups, i.e., the hydroxyl and carboxyl termini, which restrict the efficacy of fluorophore labeling. In a typical example, a labeling efficiency of 65% was achieved when 5-amino fluorescein was reacted with PLGA. Other routes to functionalized PLGA have required multiple steps and were performed in organic solvents, which in turn render scale-up challenging.

Our motivation was to overcome fluorophore leakage and the low efficiency of PLGA labeling through a simple, solvent free, one-step reaction to prepare fluorescent polymers with a label on each chain. This was achieved using fluorophores with active nucleophilic moieties as initiators in a solvent-free ring-opening polymerization (ROP) reaction of lactide (LA) and glycolide (GA) monomers. We used this method to synthesize three different fluorescently labeled PLGA copolymers, by ring-opening LA and GA in the presence of 2-aminoanthracene (2AA, blue), 5-amino fluorescein (5AF, green) and Rhodamine-6G (R6G, red). The newly synthesized polymers were then used to fabricate nanoparticles via a solvent displacement method.

The initial one-step synthesis used a conventional (Sn(Oct)$_2$) catalyzed ring opening polymerization route and fluorophores (2AA, 5AF and R6G) with pendant amines were used as the nucleophiles. The mechanism of this reaction occurs through anionic ROP, in which the initial dye amine attacks a carbonyl group in the LA or GA ring. This frees a nucleophilic hydroxyl group which opens further LA and GA rings, such that the reaction continues until all the LA and GA are consumed. The resulting polymers were denoted as PLGA-2AA, PLGA-5AF, and PLGA-R6G (Figure 1), and their structures were confirmed using $^1$H NMR spectroscopy (Figures S1, S2, and S3, respectively). The kinetics of the reaction (for %AFPLGA, as a model material) was followed and analyzed (Figure S4). These experiments showed that high conversion to polymer ratio was reached after 60 min of reaction (Figure S4A and B). By leaving the reaction for an additional 2 h, the conversion reached a plateau value, and the linearity was lost (Figure S4C). In the case of 2AA-initiated ROP reaction, a concurrent loss in molar mass control and also a drop in final yield in polymer were observed. In fact, when 2AA was adopted as an initiator after 60 min, a $D$ of 1.3 was registered (Table 1), while a $D$ of 1.8 was reached after 180 min. On the other hand, a constant value of 1.2 was observed.

Table 1. Labeled PLGA (Blue, Green, and Red) Synthesis, Composition (Monomer Ratio), Molecular Weight (g mol$^{-1}$), and Yield

| polymer     | feed ratio (mol %) | product ratio (mol %) | molecular weight (g mol$^{-1}$) | yield (%) |
|-------------|--------------------|-----------------------|---------------------------------|-----------|
|             | LA | GA | dye | LA | GA | dye | $M_{n}$ | $M_{w}$ | $D$  |
| PLGA-2AA    | 59 | 39 | 2   | 46 | 32 | 2   | 15000 | 5500  | 7260 | 1.3 | 65% |
| PLGA-5AF    | 59 | 39 | 2   | 47 | 40 | 2   | 11600 | 8700  | 10440 | 1.2 | 65% |
| PLGA-R6G    | 59 | 39 | 2   | 53 | 33 | 2   | 11900 | 5600  | 7840 | 1.4 | 60% |

| a NMR-calculated molar mass ($M_{n}$ (calcd)) and determined molar masses ($M_{n}$, $M_{w}$ via GPC, using PMMA standards as calibrants) and polydispersity index ($D$). LA = lactide, GA = glycolide.

![Figure 1. Schematic representation of PLGA-2AA, PLGA-5AF, PLGA-R6G, and synthesis using ROP. All reactions were conducted at 140 °C under nitrogen (N$_2$), catalyzed by tin(II) 2-ethylhexanoate (Sn(Oct)$_2$).](https://dx.doi.org/10.1021/acsmacrolett.9b01014)
for SAF-initiated PLGA independently from the reaction time. This may imply an increasing instability with time, under reaction conditions, and triggering side reactions (unzipping or branching) in the presence of 2AA. All the synthesized polymers showed a narrower molecular weight distribution compared to the commercially available labeled PLGA ($\bar{D}$ $\sim$ 1.55, Figure S5). Moreover, the presence of the dye linked to the polymeric chain was confirmed by GPC measurements carried out with UV−vis−RI tandem detectors. The two traces overlapped for all the synthesized polymers confirming the presence of the dye on the polymer chain (Figure S5 and insets).

The yields of purified polymers for PLGA-AA, PLGA-5AF, and PLGA-R6G were 65% (gray solid), 65% (deep orange solid), and 60% (light pink solid), respectively (Table 1). Following synthesis, PLGA nanoparticles were produced via a controlled flow solvent/antisolvent nanoprecipitation methodology. Both fluorescent and unlabeled PLGA NPs had comparable hydrodynamic diameters, centered at 100 nm, and with zeta potentials of approximately $-40$ mV (Table S1). TEM imaging confirmed that the fluorescent nanoparticles were of low size dispersity and that there were no obvious differences between the polymers used to manufacture the nanoparticles (Figure S6), which is in accord with previously reported literature. To compare the relative emission intensities of newly prepared fluorescent-dye-initiated PLGA nanoparticles vs unlabeled PLGA nanoparticles loaded with the analogous dye (5-AF), fluorescence spectroscopy was conducted. PLGA-5AF nanoparticles were found to be $\sim$12.5 X brighter than PLGA nanoparticles loaded with 5-AF ($p < 0.0001$). Furthermore, 24 h after initial preparation, the fluorescence of PLGA-5AF nanoparticles had decreased by $\sim$3% whereas for PLGA nanoparticles loaded with 5-AF, the fluorescence intensity reduced by 45% (Figure S7A). These

![Super-resolution microscopy images of fluorescent nanoparticles](image-url)
results indicated PLGA nanoparticles prepared using fluorophore linked PLGA were significantly brighter \((p < 0.0001)\) and demonstrated enhanced stability after 24 h of storage, thus suggesting their promise for medium and long-term experimentation. In addition, 100 nm nanoparticles prepared from 5-amino-fluorescein were ∼8-fold brighter than a commercially available fluorescent-labeled PLGA formulated into analogous 100 nm particles (Figure S8). It is also important to note that AF, a fluorescein, is a pH-sensitive fluorophore. As such, the fluorescence intensity of fluorescein derivatives is quenched in acidic environments,\(^{17,23}\) such as those found in endosomal/lysosomal compartments.\(^{24}\) Therefore, observation of live cell behavior with PLGA-AF polymeric nanoparticles, in the absence of a ratiometric control, can be challenging. This effect can be overcome through chemical fixation and resuspension of cells for analysis in buffered media, such as phosphate-buffered saline (pH 7.4), to equilibrate cellular pH where AF fluorescence intensity is not diminished (please see SI).\(^{25}\)

The effects of these PLGA nanoparticles on cell metabolism were then evaluated through AlamarBlue assays and the intracellular location of internalized particles further investigated with wide field fluorescence microscopy in human T-helper-precursor-1 (THP-1) macrophages, a model cell line for determining in vitro biocompatibility and particle uptake.\(^{25}\) These assays directly compared PLGA NPs (PLGA-AA, PLGA-AF and PLGA-R6G), with commercially produced unlabeled PLGA (PLGA), fluorophore loaded PLGA (AF loaded NPs) and a commercially available green fluorescent PLGA. Cells were challenged with increasing nanoparticle concentrations \((50–500 \mu\text{g/mL})\) for 24 h.

Fluorescently labeled PLGA NPs were well-tolerated by the cells, as more than 75% of cells were shown to be actively metabolizing at 500 \(\mu\text{g/mL}\) (Figure S7B). More importantly, no significant difference was observed in cell viability between PLGA-AA, PLGA-AF, PLGA-R6G, PLGA (commercial), AF loaded PLGA NPs and commercially available dye-conjugated nanoparticles at all experimental concentrations \((p < 0.01)\).

Therefore, a concentration of 100 \(\mu\text{g/mL}\) was used for subsequent experiments.

To investigate the subcellular localization of fluorescently labeled PLGA NPs (100 \(\mu\text{g/mL}\)), THP-1 cells were dosed with particles for 3 h and imaged using super-resolution confocal microscopy. All fluorescent PLGA NPs were internalized as demonstrated by the orthogonal cross sections (Figure 2Ai–Ci). As apparent from Figure 2A–D, dye-initiated PLGA nanoparticles (PLGA-AF) demonstrated rather different fluorescence responses, when compared to fluorophore-loaded PLGA nanoparticles. Images A, B, and C show the dye-initiated nanoparticles localized in bright fluorescent regions, whereas Figure 2Di indicated that the dye-loaded particles were less bright and more diffuse in the cells. For Figure 2Di and 2Dii, the same regions of interest are shown; however, Figure 2Di was acquired under the same experimental laser power as Figure 2Bi, whereas Figure 2Dii was acquired at 8 times the laser power in order to excite the same level of fluorescence as occurred from the dye-initiated nanoparticles in Bi.
In addition, the images shown in Figure 2Aii, Bi and Cii indicated that the dye-initiated nanoparticles were present in discrete subcellular vesicles. This suggested that the dyes were retained with the polymers in nanoparticles which were stable to endocytic pathways and intracellular transport processes. In contrast, close analysis of Figure 2Dii and 2Diii revealed no clear punctate regions, implying that the PLGA NPs loaded with AF released their contents into a diffuse intracellular space and that dye and nanoparticles were most probably no longer colocalized. These data were further corroborated with analysis of the dye-initiated PLGA nanoparticles in comparison with the commercial dye-conjugated 100 nm PLGA nanoparticle formulation. As apparent in Figure S9, both nanoparticle formulations were localized in punctate regions in the THP-1 cells, again indicating that covalent attachment of dyes to the polymer chains prevented general cytosolic fluorescence due to generalized dye diffusion. The dye-initiated PLGA nanoparticles were also notably brighter than the commercial samples (Figure S9), as evidenced by comparison of the images optimized for brightness for each nanoparticle type.

In order to demonstrate the advantages of the single-chain fluorescently labeled PLGA (PLGA-AF) for tracing the intracellular fate of nanomaterials in a representative drug delivery experiment, the polymers were formed into nanoparticles containing encapsulated doxorubicin hydrochloride. This DNA intercalating anticancer drug exhibits red fluorescence (λ_ex = 480 nm, λ_em = 590 nm) and has been very widely used both in laboratory studies and in the clinic.26-29 We selected THP-1 cells for these experiments because they are nonproliferative, which makes them tolerant to doxorubicin and therefore a good model to study its cellular delivery. Accordingly, doxorubicin-loaded PLGA-AF NPs were fabricated and dosed to activated THP-1 cells at subtoxic concentrations (100 μg/mL, 3 h).

Figure 3 shows that doxorubicin fluorescence (red) was distributed in regions of interest away from PLGA-AF (green) when imaged after 3 h (Figure 3A) and after 24 h (Figure 3B). Through further analysis of microscopy images, the data suggest that doxorubicin/PLGA-AF formulated nanoparticles (yellow) appear to reside in punctate vesicles, which were likely to be lysosomal or endosomal compartments. Doxorubicin was found to diffuse away from these regions and the labeled polymers, while the aminofluorescein dye remained attached to the polymers over these time periods. However, the extent of any degradation of the PLGA chains during the cell assays could not be ascertained other than that no fragments were able to diffuse away at the same rate as the small-molecule drug.

The release of doxorubicin from PLGA-AF nanoparticles was confirmed in parallel release assays in aqueous media: after 24 h the fluorescence intensity of PLGA nanoparticles loaded with doxorubicin by nanoprecipitation had dropped by 44% (Figure 3C). These results highlighted the advantages of dye-initiated PLGA during the monitoring of therapeutic delivery to show the subcellular distribution of both drug and carriers in vitro.

The culminating experiments aimed to demonstrate the advantages of the single-chain-labeled PLGA in vivo. The nematode Caenorhabditis elegans was chosen as an accessible model to study in vivo biodistribution of the labeled nanoparticles (Figure 4). Synchronized cycles of C. elegans (L4-young adult stages) were washed with sterile deionized water prior to dosing, and ~300 nematodes were then exposed to a range of PLGA nanoparticles at 100 μg/mL (n = 3) for 3 h. The nematodes were washed again before examination and collected for fluorescence microscopy imaging.

The images in Figure 4 clearly show the internalization of the nanoparticles and their localization in the digestive tracts of the nematodes. The key result in these experiments was that the different localization of fluorescence arising from the dyes encapsulated in the nanoparticles and the fluorescence from the dyes covalently linked to the PLGA chains. The encapsulated rhodamine B dye diffused throughout the nematode digestive tract within 12 h (Figure 4B) and was...
not fully colocalized with the fluorescein signals from the nanoparticles (Figure 4D,E). For the dye-initiated NPs, bright fluorescence was apparent in discrete regions of the nematodes (Figure 4G,H), and this was retained throughout the experiment without any obvious changes in the behavior or viability of the worms (Figure S10). These experiments indicated good tolerability of the nanoparticles in addition to the retention of their bright fluorescence in vivo. In addition, and of crucial importance, the data indicated the advantage of using covalently linked dyes, in which every polymer chain was tagged, to track in vivo transport of nanoparticles in the presence of encapsulated labels and/or actives.

This study described the synthesis of fully fluorescently labeled PLGA using three different fluorophores via a single-step ROP reaction under solvent-free conditions. The resultant dye-initiated PLGA polymers were able to self-assemble into well-defined nanoparticles which were significantly more bright in fluorescence microscopy when compared to dye-encapsulated and commercially available dye-linked analogues. The nanoparticles were readily internalized by activated THP-1 macrophages and exhibited a comparable cytocompatibility profile to commercially available PLGA in vitro. In addition, the stability of the fluorescent dye–protein link was demonstrated by retention of the label in punctate regions of the THP1 cells, again in contrast to dye-encapsulated PLGA, which released the entrapped label over time. In turn this property was exploited in a model drug delivery experiment in which the separate intracellular locations of a released drug, doxorubicin, and the carrier polymers were shown in THP-1 cells. The preliminary in vivo study in C. elegans further exemplified the use of these bright and stable nanoparticles, with clear differences between the distribution of released dye and dye-initiated PLGA nanoparticles. We believe that this synthetic strategy may be further used to prepare a range of intrinsically labeled biodegradable polymers which could greatly facilitate the tracking of delivery systems in subcellular locations in the presence of multiple therapeutic constructs. In turn, these materials will allow better characterization of delivery pathways and cellular transport kinetics to accelerate clinical translation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmacrolett.9b01014.

Full experimental methods are provided, including NMR spectra of polymers, GPC chromatograms, TEM, DLS, and zeta potential data, and details of cell and nematode culture (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

All raw data created during this research are openly available from the corresponding author cameron.alexander@nottingham.ac.uk and at the University of Nottingham Research Data Management Repository (https://rdmc.nottingham.ac.uk/), and all analyzed data supporting this study are provided as Supporting Information accompanying this paper.

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