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Development and Analysis of Functionalized Poly(Lactide-Co-Glycolide) Polymer for Drug Delivery

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Abstract

Functionalized polymer molecules are the backbone for formulation of targeted biomolecule delivery systems. We report here an effective method for the chemical synthesis of poly (lactide-co-glycolide)-1,4 diaminobutane (PLGA-DAB) in a controlled manner and an efficient gel permeation chromatography (GPC) method for analysis of functionalized PLGA. The synthetic method is simple, reproducible and the functionalized PLGA can be conjugated with any carboxylic acid-containing ligand and ultimately used for targeted drug delivery. The developed GPC analytical method is accurate, selective, precise and easily adaptable for analysing any other functionalized polymer with an amine group. These techniques provide an important basis for formulation scientists to develop functionalized polymers for targeted nanoparticle drug delivery.

Keywords: Functionalised PLGA; Conjugation; Carbodiimide cross-linking; GPC; Fluorescence Spectrophotometry; Fluorescamine; Primary amine analysis; Non-aqueous

Introduction

Poly (lactide-co-glycolide) (PLGA) copolymers are biocompatible and biodegradable, and thus widely used by formulation scientists for drug delivery. They are approved by the US Food and Drug Administration (FDA) for use in parenteral drug delivery systems [1]. PLGA has versatile and adaptable characteristics that can be controlled by the degree of crystallinity, ratio between the two core monomers (lactic acid/glycolic acid), and the total molecular weight. The polymer is available with different end groups (for example, with free carboxylic end group, ester-terminated group etc.) allowing choice of the most applicable polymer based on potential chemical reactions and modification requirement.

PLGA is often used as a backbone polymer to construct smart drug delivery systems, such as nanoparticles (NP) to carry, protect, and target their payload to a specific site in the body in an efficient manner. PLGA nanoparticles have shown promising results when used as carriers for drugs, peptides, proteins, vaccines, and nucleotides [2-6]. These nanoparticles protect drug moieties from degradation by various body systems, and can also be designed to provide sustained drug release, facilitate intracellular delivery of bioactive materials, accumulate within target tissues (e.g., tumour) and even with potential to penetrate the blood-brain barrier (BBB) [7]

However, PLGA NP cannot satisfy the ultimate goal of enhanced and targeted biomolecule delivery as they are rapidly cleared from the systemic circulation following intravenous administration. In addition, without effective targeting ligands PLGA nanoparticles cannot differentiate between normal and diseased cells to facilitate enhanced delivery to the required site to generate a therapeutic action. Finally, negatively charged PLGA NP have limited cellular uptake and are ineffective to cross tight junctions present in the reach the target inside the brain [8,9].

Modification of the PLGA polymer to produce surface functionalized NP can overcome these challenges and improve *in vivo* performance of NP [10]. Functionalized PLGA NP can exhibit prolonged systemic circulation time, enhanced accumulation at the intended site of action, and where applicable, improved penetration across the BBB. These capabilities can reduce the required drug dose and improve the safety profile of the applied drug.

There are a number of reports on how to functionalize PLGA with various small molecules [8,9], however the challenge remains to conjugate PLGA to small molecules with bi-functional groups in a controlled manner and accurately quantify polymer conjugates, especially from the crude reaction mixture [11,12]. We have developed an effective approach for functionalising PLGA, and a GPC method that can be used during the polymer conjugation stage for monitoring the reaction progress (crude reaction mixture and the final product).

Fluorescamine (4-Phenylspiro-[furan-2(3H),1-phthalan]-3,3′-dione) is a widely-used reagent for the analysis of primary amine groups, as it produces highly fluorescent pyrrolidone derivatives [13,14]. Fluorescamine is not fluorescent itself but can react with a primary amine at room temperature, resulting in a fluorophore with strong intensity in both aqueous and non-aqueous systems [15]. However, it has a narrow suitable pH range (7.5 to 9.0) in aqueous systems and the reaction completion time is unpredictable in non-aqueous systems [15,16]. There is added challenge for its application in analysis of functional groups in polymer due to the issue of accessibility and solubility of the functional groups in selected solvents. These challenges and drawbacks render the need for development of effective fluorescamine derivitization based assays for many functionalized polymers, including polymer-based delivery systems. The first aim of the current study was to chemically conjugate PLGA copolymers with diaminobutane (DAB) to create a reactive intermediate (PLGA-DAB) that can facilitate further ligand attachment, or can act as cationic PLGA polymer. PLGA-DAB, as an intermediate, can be easily further modified to react with carboxylic acid containing targeting ligands such as amino acids, folic acids, sialic acid, hyaluronic acid etc. We proposed the use of Boc-DAB to functionalize PLGA as DAB is a simple and widely available small molecule with dual amine groups. Boc-DAB is a form of DAB with one side amine group protected, which allows control of the conjugation process. The resultant PLGA-DAB is a versatile polymer, as it is both a functional polymer and a building block for other sophisticated smart polymers via carbodiimide conjugation.

The second aim was to develop an appropriate quantification method that can separate the polymer-amine conjugate from other impurities, and quantitatively analyse the functionalised polymer with good accuracy and selectivity. Similar studies have been reported for primary amine analysis in both aqueous and non-aqueous systems, by using fluorescence

spectrophotometry [17]. However, there is no suitable method available to quantify small amine molecules attached to a relatively large polymer molecule during a reaction. This type of analysis requires an optimized fluorescamine derivatization process and size exclusion chromatographic method to separate and quantify the polymer-amine conjugate from possible impurities.

Experimental

Materials

Acid terminated poly(lactide-co-glycolide) (PLGA, intrinsic viscosity 0.19 dL/g, number average molecular weight ~3.5 kDa), with a 50/50 ratio (polylactic acid/polyglycolic acid) was supplied as LACTEL Absorbable Polymers (Durect Corporation, Birmingham, USA); N-Boc-1,4-diaminobutane (Boc-DAB), Fluorescamine, Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS), Trifluoroacetic acid (TFA) and Triethylamine (TEA) were purchased from Sigma-Aldrich (Castle Hill, Australia). Tetrahydrofuran (THF) and acetonitrile (ACN) were obtained from Thermo Fisher Scientific (Scoresby, Australia). Deuterated dimethyl sulphoxide(DMSO-D6) containing 0.05% v/v tetramethylsilane (TMS) was procured from Cambridge Isotope Laboratories, Inc. (Andover, USA). All other analytical reagents were of highest possible purities. Polystyrene standards were supplied by Shodex® (Tokyo, Japan). Millex-HN Syringe-tip filters were from Merck Millipore (Bayswater, Australia).

Synthesis of poly(lactide-co-glycolide)-diaminobutane conjugate (PLGA-DAB)

PLGA-DAB was synthesized in three steps (Fig. 1) following modification of reported methods [18-20]. Carbodiimide cross-linking was achieved using DCC/NHS as catalyst, which mediated the formation of an amide linkage between the terminal carboxylic group of PLGA and free amine group of Boc-DAB. The Boc group was then removed to yield the final product, PLGA-DAB. The process is summarized below.

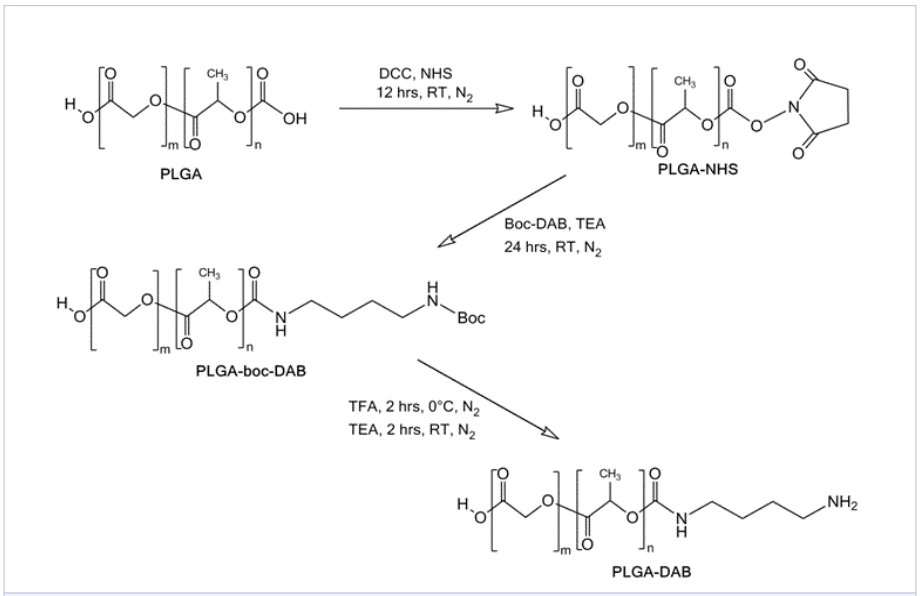

Figure 1: Scheme for conjugation of PLGA and DAB using DCC/NHS based coupling

Activation of -COOH group on PLGA

Briefly, PLGA (175 mg, 0.05 m mole), DCC (52 mg, 0.25mmole) and NHS (29 mg, 0.25mmole) were dissolved in 10mL ACN and stirred for 12 hrs at room temperature under nitrogen environment. Completion of this reaction was determined by hydroxamate method, described separately in section 2.3. The reaction mixture was filtered through a 0.4µm nylon syringe-tip filter to remove the insoluble dicyclohexylurea (DCU) by-product. The activated PLGA was isolated by precipitation into 100 mL methanol under vigorous stirring. The clear supernatant was discarded upon centrifugation at 2000g for 20 min. The precipitate was washed with cold methanol 3-4 times to remove unreacted DCC and NHS and then dried in a vacuum oven at 30°C for 10 min.

Synthesis of PLGA-boc-DAB

Boc-DAB (two equivalents of PLGA), was slowly added drop wise to the solution containing activated PLGA and TEA (20 equivalents of Boc-DAB) in 10mL ACN and kept on a stirrer for 24 hrs at room temperature under nitrogen environment. TEA acted as a catalyst to yield permanently bonded amide conjugate [18]. The reaction mixture was precipitated in diethyl ether under vigorous stirring and subsequently centrifuged at 2000g for 20 min to collect PLGA-Boc-DAB conjugate. The resulting pellet was washed 2-3 times with diethyl ether to remove unreacted Boc-DAB and TEA. The product was dried in a vacuum oven at 30°C for 60 min. The percentage of Boc-DAB conjugated to PLGA was determined by ^{1}H NMR spectroscopy, described separately in section 2.4.

Removal of Boc-group

100mg of PLGA-Boc-DAB conjugate (0.03mmole of Boc-DAB) was dissolved in 5mL dichloromethane (DCM), 500 molar equivalent pure TFA was added to the solution and stirred at 0°C for two hrs under nitrogen environment. DCM and TFA were removed under vacuum at 40°C in the rotary evaporator (Büchi Rotavapor R-200, Switzerland). The solid mass was redissolved in DCM and then evaporated using similar settings. This was repeated 8-9 times for complete removal of free TFA. The resulting PLGA-DAB-TFA salt was treated with TEA for two hrs at room temperature at a salt:TEA molar ratio of 1:10 to produce PLGA-DAB. The mixture was placed in a dialysis bag (MWCO 2000Da) and dialyzed for 12 hrs against Milli-Q water using a dynamic flow of 1mL/min. Finally, the content of the dialysis bag was frozen at −40°C and lyophilized for 48 hrs in a Dynavac FD3 freeze dryer (John Morris, Perth, and Australia) to acquire a solid product.

Determination of degree of PLGA activation by hydroxamate method

Hydroxamate method was used to determine the degree of PLGA activation, as previously reported [19,20]. This method is based the principle of analysing free NHS released from activated PLGA-NHS. Briefly, 0.2mL of 2N sodium hydroxide (NaOH) was added to 1mL of PLGA-NHS solution (in between 1 and 10 mM) to produce free NHS by breaking down the PLGA-NHS conjugate. The reaction mixture was placed in a water bath at 40°C. After 10 min, it was acidified by adding 1.5mL of 0.85N hydrochloric acid (HCl) and centrifuged to remove precipitated polymer. 0.5mLdeveloping solution (5% ferric chloride in 0.1N HCl) was mixed with 2mL of the supernatant to produce a brown colour. The intensity of the developed colour represented the degree of PLGA activation. A standard curve was prepared with five known concentrations of NHS (1mM to 10 mM, 1 mL each) processed/analysed by the same procedures. A UV-vis spectrophotometer (Shimadzu UV mini-1240 series, Shimadzu Corporation, Japan) was used to measure absorbance at 500 nm. The unreacted NHS in PLGA-NHS solution was quantified directly without addition of NaOH by the same method.

Characterization of PLGA-Boc-DAB conjugate by ^{1}H NMR spectroscopy

^{1}H NMR spectra were recorded for both the starting materials (Boc-DAB and PLGA) and PLGA-Boc-DAB conjugate by NMR spectrometer (Bruker Ultrashield Plus Biospin GmbH Nuclear Magnetic Resonance Spectrometer, Avance III 400 MHz, NaNoBay, Bremen, Germany) using DMSO-D6 as the solvent and TMS as the internal standard.

Quantitation of PLGA-DAB by GPC

The starting material (Boc-DAB) and the final product (PLGA-DAB) were analysed by gel permeation chromatography (GPC), which is capable of separating and quantifying a molecule of interest from the polymer mixture. To accurately quantify PLGA-DAB, we coupled fluorescamine to PLGA-DAB and conducted analysis by GPC with a fluorescent detector (FLD) and refractive index detector (RID). To develop the most suitable

method for analysing PLGA-DAB, we examined both the method developed by Eckstein and Dreyfuss for the spectrophotometric determination of DAB in chloroform [17] and another method published by Tan and Patsiga [15]. Method one was unsuitable because Boc-DAB was insoluble in chloroform. ACN was therefore used to dissolve Boc-DAB and PLGA-DAB. 1mL of this solution was mixed with 1mL of 2 mM fluorescamine aqueous solution. The mixture was thoroughly mixed with the aid of a Vortex stirrer for 2min, then 2 mL of 95% ethanol was added to bring the reaction between fluorescamine and the amine to completion, and vortexed for a further 8 min. Fluorescence spectra were measured with Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies Australia, Mulgrave) using excitation/emission wavelengths of 390 nm/473 nm. The mixture was analysed immediately, and every hour for eight hours, to determine whether the ethanol was bringing the reaction between fluorescamine and the amine to completion.

We found that ethanol was ineffective to complete the derivatization process. Method two, a non-aqueous method, was developed based on the published method by Tan and Patsiga, with some modification [15]. Briefly, 1 mL solution of Boc-DAB(200 ng/mL or 1.1 μM) or PLGA-DAB(1.1 μM) in acetonitrile was mixed with 0.5 mL TEA (70 nM) and vortexed for 2min to obtain a homogeneous basic solution. 1.5 mL of 2mM fluorescamine in ACN was then added to the mixture and further vortexed for 23 min for the reaction to be completed. The mixture was analysed by fluorescence spectrophotometry.

Optimum basicity, which is critical for the fluorescamine derivatization step, was determined using different TEA solutions from 70 picomolar to 0.7 molar (7×10^{-11} to 7×10^{-1}M), mixed with each mL of the same Boc-DAB standard. The mixtures were vortexed for 2 min, then 1.5 mL fluorescamine (2 mM) was added to each mixture, followed by vortexing for 23 min. Samples were analysed by fluorescence spectrophotometry. To determine the effect of derivatization time on analysis, Boc-DAB standard was derivatized using TEA/fluorescamine (method two). From derivatization start point (addition of TEA), fluorescent intensity was measured frequently up to 1 hour (5, 10, 15, 20, 25, 30,45 min), and then after every hour to 24 hours.

Once optimum conditions were established, GPC was used to analyse both Boc-DAB and polymer-DAB conjugates after conversion to their fluorescent derivatives with fluorescamine. Agilent 1200 system (Agilent Technologies Australia, Mulgrave) consisted of a degasser (G1379B), binary pump (G1312A), auto sampler (G1329A) with thermo-control unit (G1330B), and thermostat controlled column compartment (G1316A), fluorescence detector (G1321A) and refractive index detector (G1362A). Acquired data were processed by Agilent ChemStation®, B.04.03 SP1 software. The GPC column was a Jordi Gel DVB, 100-50000Da molecular weight range, 5 μm particle sizes, 1000 Å pore size, 250 mm × 4.6 mm ID, with a Jordi 5μm mixed bed GPC guard column, 50mm × 4.6mm ID (Mansfield, USA).

The mobile phase was 100% THF at flow rate 0.5 mL/min. Boc-DAB standards were prepared in acetonitrile. Upon derivatization with fluorescamine, samples were filtered and collected through a 0.45 μm nylon syringe-tip filter after discarding the first 2 mL of the filtrate. All samples were analysed by duplicate injection of 50 μL with needle wash by the mobile phase. Polystyrene standard solutions (3.79 kDa to 956 kDa) from Shodex® (F8602105, standard SM-105) in THF were prepared as per manufacturerinstruction and used as the column quality control. Calibration curves were obtained using 25, 50, 100, 200 and 250 ng/mL of Boc-DAB standard solutions in acetonitrile, maintaining the basic pH with 70 nM TEA. PLGA-DAB samples were prepared in acetonitrile and analysed as per Boc-DAB. In addition, crude reaction mixtures in acetonitrile were also analysed after filtering through a 0.45 μm nylon syringe filter.

ICH guidelines for Validation of Analytical Procedures, Q2B [21] were followed for validating the developed GPC method. The parameters evaluated were linearity, precision, accuracy, selectivity, limit of detection (LOD), limit of quantification (LOQ), and reproducibility.

Linearity was measured by analysing five concentrations 25, 50, 100, 200 and 250 ng/mL of both Boc-DAB standard solutions and PLGA-DAB samples. Test results were evaluated by calculation of regression line by the method of least squares and reported as the square value of correlation coefficient (R^2).

Accuracy was established by analysing the reaction mixture and the reaction mixture which was spiked with Boc-DAB standard at three concentration levels (25, 100, and 250 ng/mL). Percentage recovery from three replicates was calculated to assess the accuracy of the method. Accuracy values of between 90 and 110% were considered acceptable.

Precision was determined as repeatability and intermediate precision. The intra-day precision (repeatability) was assessed by injecting 50 and 200 ng/mL Boc-DAB and PLGA-DAB six times at different times in a day. Relative standard deviation (%RSD)values for both samples were calculated for which the acceptable range was up to 5.0%. The inter-day repeatability (intermediate precision) was determined by injecting the same standards six times on two consecutive days. The intra- and inter-day repeatabilities were calculated as the coefficient of variance (CV) and reported as the absolute value of the CV as percentage, i.e., %RSD.

Selectivity of the method was evaluated by making comparison among analyses of a) reaction mixture, b) reaction mixtures piked with Boc-DAB standard and c) reaction mixtures piked with PLGA-DAB. Peak purity was also checked to ensure that the analyte chromatographic peak is purely and free of possible impurities.

Limit of detection (LOD) and ***limit of quantification*** (LOQ) were calculated from the noise level in the chromatogramsat retention times of Boc-DAB and PLGA-DAB obtained from the matrix blank (reaction mixture without DAB) and detector response to both Boc-DAB standard and PLGA-DAB samples. Also, the LOD and LOQ values were determined by progressive dilutions of the lowest concentration employed for the calibration curve (25 ng/mL).

Reproducibility of the method was confirmed by running the assay in two different laboratories.

Result and Discussion

Synthesis and characterization of PLGA-DAB conjugate

Zero length cross-linkers, DCC and NHS were used for PLGA-DAB conjugate synthesis. It is a widely employed carboxylic group activation method and the formed active esters are suitable for coupling with primary amines [22]. The method involves activation of PLGA terminal carboxylic acid by DCC and NHS, and the formed O-acyl derivative of carboxylic acid readily reacts with a nucleophile like amine group of Boc-DAB. This leads to amide bond formation between Boc-DAB and PLGA. Since Boc-DAB has one end amine group protected and leaves the other end primary amine group free, this reaction using Boc-DAB allows the specific amine group reaction and prevents any possibility of forming PLGA-DAB-PLGA or PLGA-DAB-DAB polymers.

Boc-protection was removed by the addition of an excess amount of TFA [23]. Unreacted TFA was removed by addition of DCM to form positive azeotrope, which could be removed at a temperature much lower than the boiling point of TFA (72.4°C)[24]. The process of addition of DCM was repeated multiple times for the thorough removal of TFA from the reaction mixture. Evidence of TFA fume production ensured its removal process and cessation of fume production indicated completion of the process. The PLGA-DAB TFA salt was further treated with TEA to obtain free primary amine end group. Different TFA salt:TEA ratio was tested and 1:10 ratio proved to produce maximum quantity of PLGA-DAB with terminal free amine group (Fig. 2).

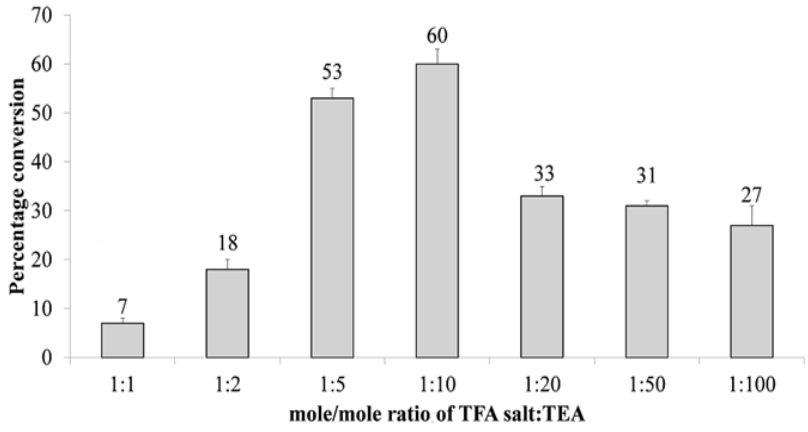

Figure 2: Effect of TEA on conversion of the PLGA-Boc-DAB TFA salt to free amine. Results are expressed as mean ± SD (n=3)

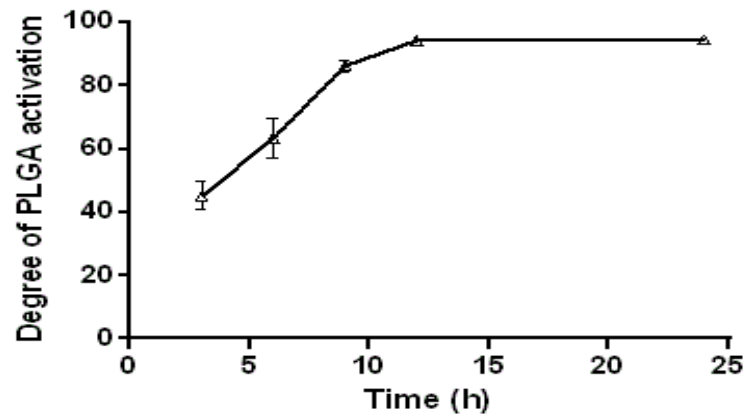

Figure 3: Effect of time on conversion of the PLGA to PLGA-NHS.
The degree of PLGA activation, as determined by hydroxamate method, was about 94% after 12 hrs of the reaction. Results are expressed as mean ± SD (n=3)

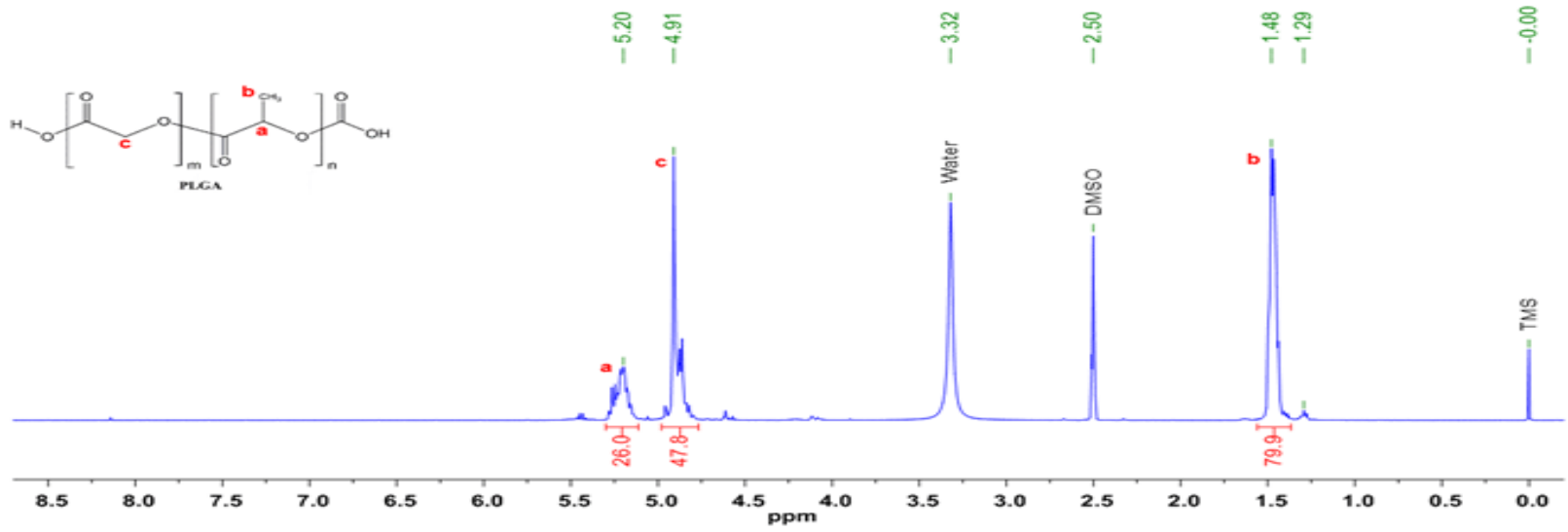

Figure 4a: [1]H NMR (400 MHz) spectra of PLGA in DMSO-D6

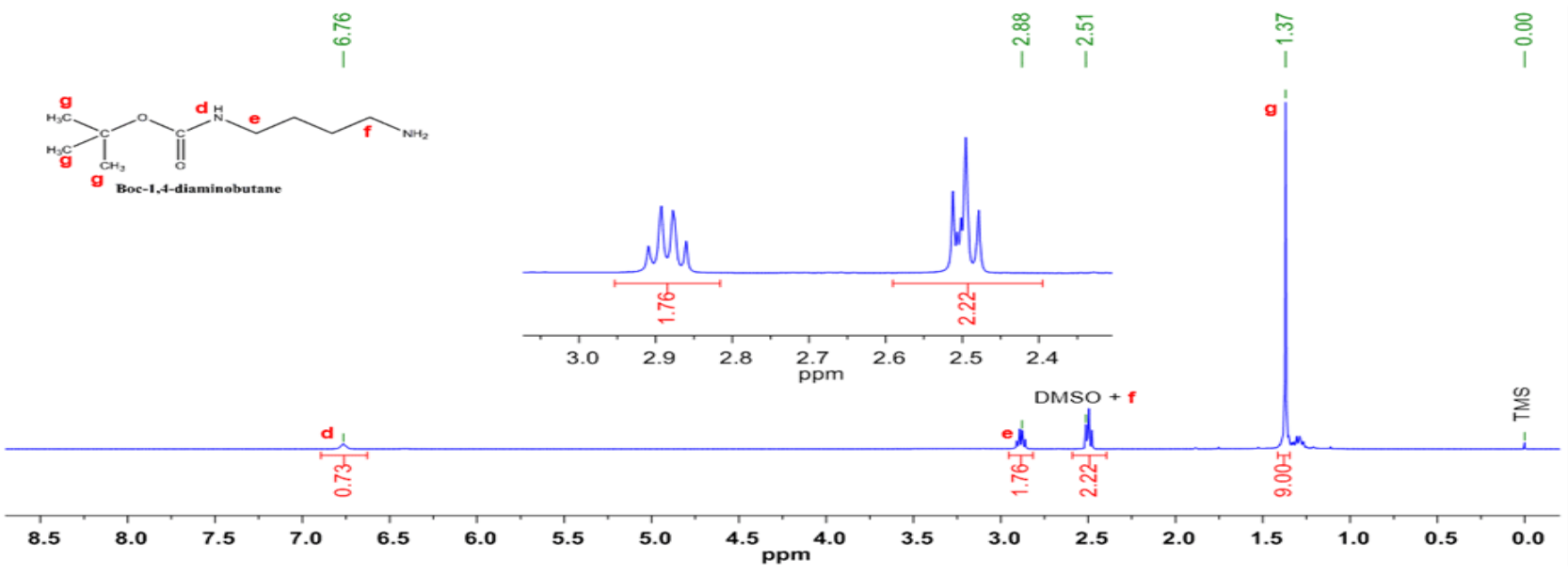

Figure 4b: [1]H NMR (400 MHz) spectra of Boc-DAB in DMSO-D6

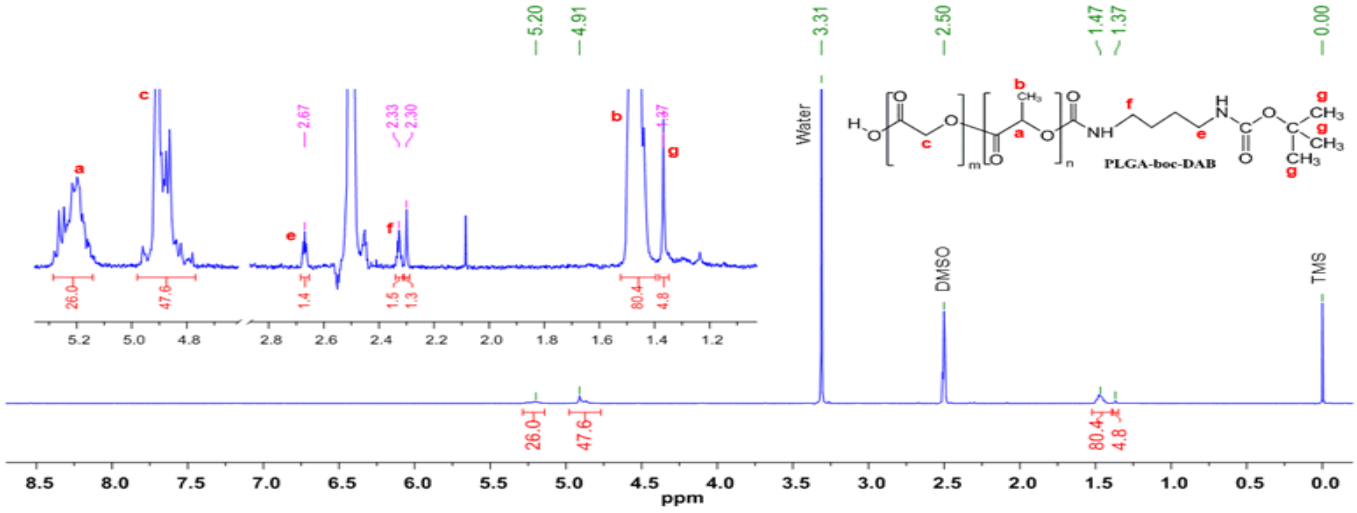

Figure 4c: ^{1}H NMR (400 MHz) spectra of PLGA-Boc-DAB conjugate inDMSO-D6

The activation of the starting molecule containing carboxyl group (-COOH) is a critical step in the carbodiimide reaction that mainly determines the final product yield. In this reaction step, the -COOH of the acid moiety forms a stable succinate ester that, upon addition of an amine compound, forms an amide. In the case of small molecules, this is a quick and straightforward reaction, but for large polymer molecules it takes a longer time to be complete. We found that the degree of PLGA activation, as determined by hydroxamate method, was about 94% after 12 hrs of the reaction (Figure: 3).

The conjugation of DAB to PLGA was confirmed by ^{1}H NMR spectroscopy. Fig. 4a, 4b and 4c show the NMR spectra of the starting materials, PLGA and Boc-DAB as well as the PLGA-Boc-DAB for the characterisation of the intermediate polymeric conjugate. PLGA-Boc-DAB spectrum clearly shows the native PLGA peaks at 1.47, 4.91 and 5.20 ppm with the correct ratio (3:2:1), and also the largest peak from native Boc-DAB (3×CH^3) is present at 1.37 ppm. The peak at 5.20 ppm was integrated as 26.0, for PLGA and PLGA-Boc-DAB, to closely match the number of protons at position 'a' in PLGA as per the product information supplied by the manufacturer. Fig. 4b shows that the -CH_2 group nearest to the Boc-protected amine group and the other -CH_2 group nearest to the free amine group in the Boc-DAB produce peaks at 2.88 and 2.51ppm, respectively. However, in the conjugate NMR spectra, these peaks moved to 2.67 and 2.33ppm position, respectively indicating that a large molecule was chemically attached with Boc-DAB and there was no free Boc-DAB present (Fig. 4c).

In Figure 4c, integration at 1.37ppm representing 3×CH_3 of Boc was found to be 4.8 when the peak at 5.20 ppm was integrated as 26.0 (i.e. representing 1 mole of PLGA). As for 1 mole Boc-DAB, it is expected to produce 9 protons at 1.37ppm, therefore the ratio of 4.8:9 indicates the extent of conjugation of Boc-DAB with PLGA (i.e. 0.53): 53% PLGA was conjugated with Boc-DAB.

Based on the NMR spectra we concluded that the PLGA polymer molecule formed an amide linkage with the Boc-DAB molecule to form the polymer-small molecule conjugate, PLGA-Boc-DAB. This conjugate formation was further confirmed by chromatographic analysis.

Optimization of fluorescamine derivatization for quantitation of PLGA-DAB

Initially, the derivatization technique outlined by Eckstein and Dreyfuss utilizing fluorescamine and ethanol to form the fluorescent DAB in chloroform was followed [17]. Due to the poor solubility of Boc-DAB in chloroform, the solvent was changed to acetonitrile, because Boc-DAB and all other reaction components had good solubility in this solvent. Ethanol was reported to facilitate the completion of derivatization reaction in a short time, but we found that there was a gradual increment of the fluorescence intensity even after eight hours of reaction (data not shown). A similar phenomenon has previously been reported [25]. This is not surprising as the ethanol itself reacts with fluorescamine to form additional products. It has been suggested that ethanol should be avoided for primary amine analysis by fluorescamine derivatization [26]. Hence, in method two we removed ethanol and raised the pH of the derivatization using TEA.

To maximize the fluorescamine derivatization,a certain level of alkalinity is required to obtain free amine groups for the formation of the fluorophore in acetonitrile. An optimum basic condition for the derivatization was established with TEA concentration range from 7nM to 7µM, therefore, a mid-point concentration of 70 nM TEA was used to maintain the basic condition for derivatization (Fig. 5 and 6).

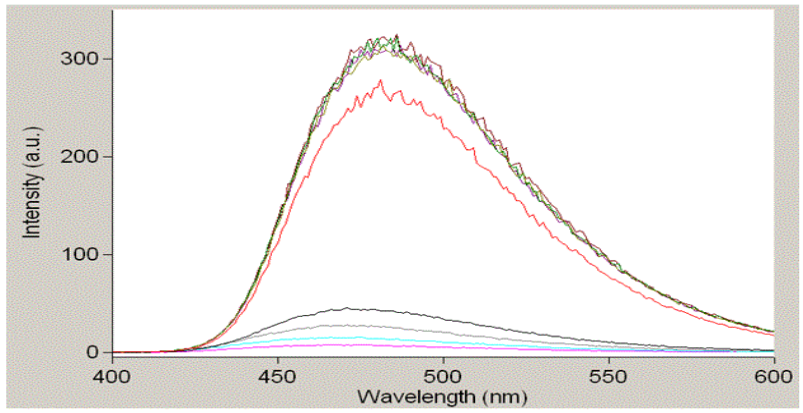

Figure 5: Fluorescence intensity (a.u.) of DAB-fluorescamine fluorophore derivatized using various concentrations of TEA

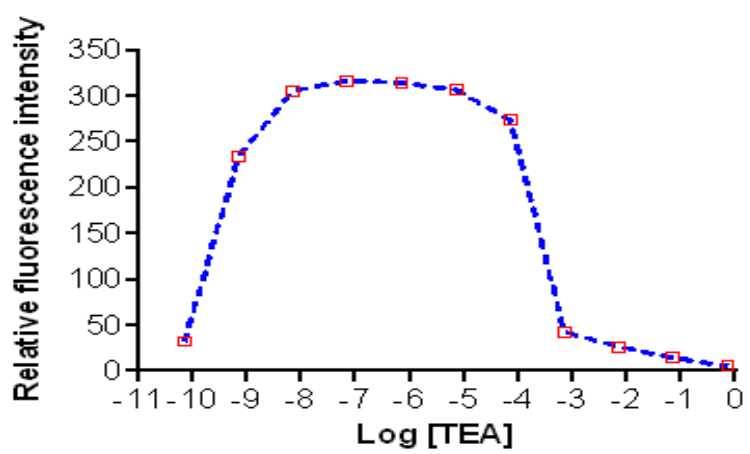

Figure 6: Optimization of TEA concentration
An optimum basic condition of the derivatization was established with TEA concentration range from 7 nM (7×10^{-9} M) to 7 µM (7×10^{-6} M).

Table 1: Effect of TEA concentration on Boc-DAB derivatization by fluorescamine

Concentration of TEA used			Relative fluorescence intensity
Conc.	Molar Conc. (M)	log [M]	
70pM	7×10^{-11}	−10.15	33
700pM	7×10^{-10}	−9.15	235
7nM	7×10^{-9}	−8.15	306
70nM	7×10^{-8}	−7.15	317
700nM	7×10^{-7}	−6.15	315
7μM	7×10^{-6}	−5.15	307
70μM	7×10^{-5}	−4.15	274
700μM	7×10^{-4}	−3.15	43
7mM	7×10^{-3}	−2.15	27
70mM	7×10^{-2}	−1.15	15
700mM	7×10^{-1}	−0.15	6

Fluorescamine derivatives are not stable and the fluorophores undergo photo bleaching [27]. Therefore, it is important to identify the time period within which analysis should be carried out to produce accurate and reproducible results. Our data of the appropriate time window for analysis of PLGA-DABfluorescamine showed that maximum fluorescence was reached at 25 min post-derivatization. The fluorescamine derivatives of Boc-DAB standard and PLGA-DAB solutions were stable for 24 hrs at 4°C (Fig. 7).

However, at room temperature, in the presence of light, the fluorescence intensity of samples reduced to below 90% within 90 min, agreeing with published data [28,29].

Based on these findings, all derivatized samples were maintained at 4°C and analysed within 24 hours. We recommend employing an autosampler with thermo-control unit at 4°C for auto injection cycles to ensure sample stability during GPC analysis.

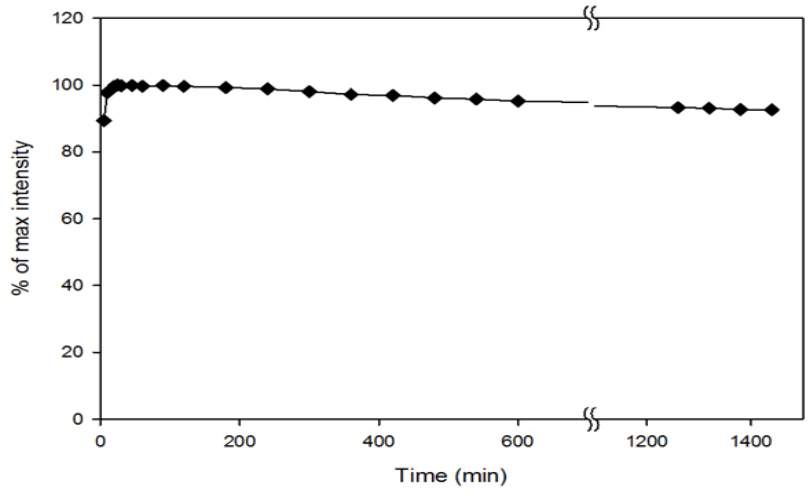

Figure 7: Effect of time on the fluorescence intensity of the sample stored at 4°C.

GPC analysis of Boc-DAB and PLGA-DAB

Chromatography and resolution

Boc-DAB fluorescent derivatives were analysed using GPC with both refractive index (RID) and fluorescence (FLD) detectors.

The Boc-DAB derivatives eluted without any interfering peaks at a retention time of 6.1 min. The analysis was performed maintaining the column and RID temperature at 30°C at a flow rate of 0.5 mL/min and the excitation/emission wavelengths of 390 and 473nm, respectively. Retention times were 6.1 and 4.8 min (FLD) for fluorescamine derivatives of Boc-DAB and PLGA-DAB, respectively. The RID confirmed that the peak at 4.8 min was a large polymer molecule (Fig. 8).

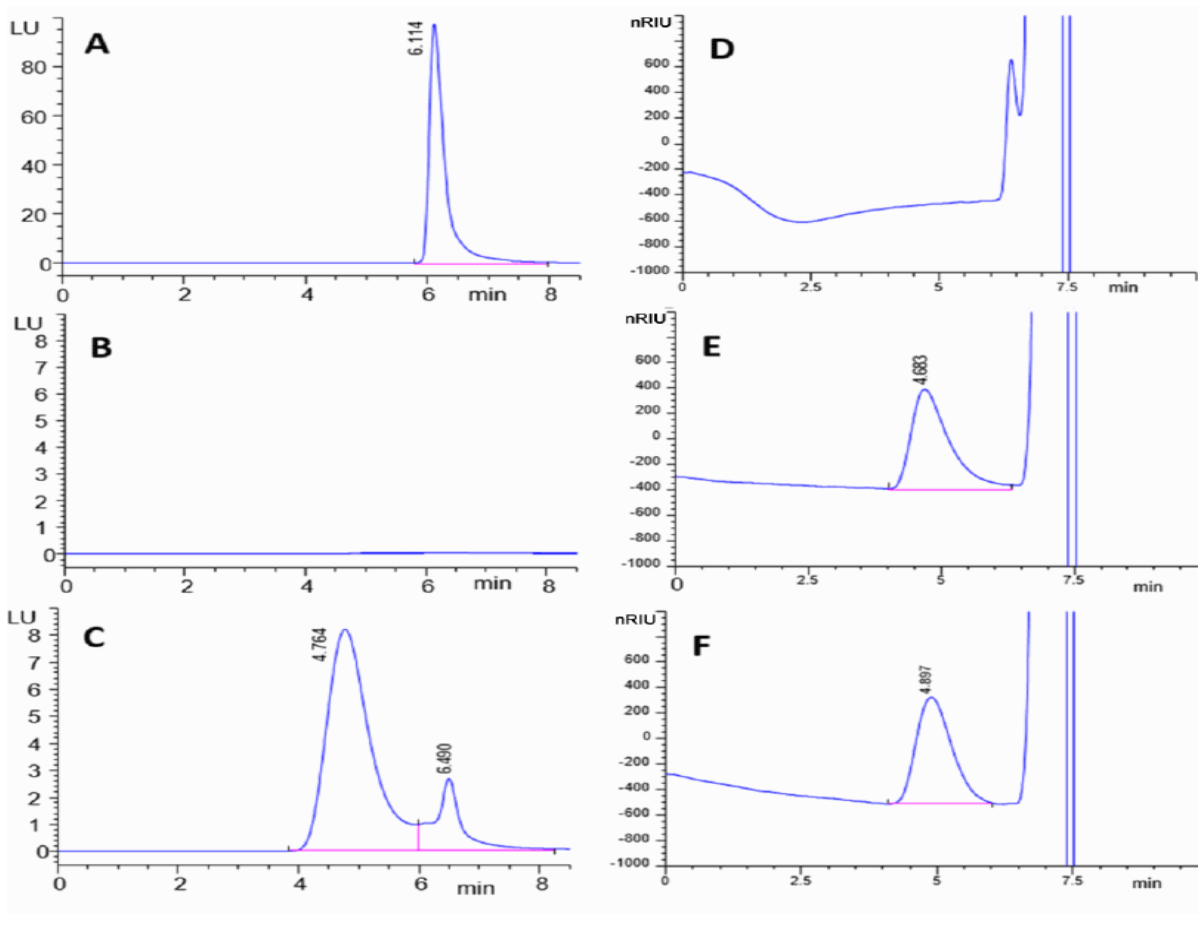

Figure 8: HPLC chromatogram of derivatized Boc-DAB (A, D), PLGA (B, E) and derivatized PLGA-DAB (C, F). The A, B, C spectra were obtained by the analysis using a fluorescent detector; the D, E, F spectra were obtained using a refractive index detector illustrating that the PLGA molecule is chemically conjugated with DAB producing PLGA-DAB

All samples were analysed by GPC using injection volumes of 50 µL. A good linearity was achieved (R^2 = 0.9992) over the range of 15 ng/mL to 50 µg/mL for Boc-DAB fluorescamine derivatives.

Validation of the GPC method

Accuracy

The method was effectively used to analyse 25, 100 and 250 ng/mL Boc-DAB standards with 106.3, 104.2 and 100.5% accuracy, respectively (Table 2).

Table 2: Accuracy data of the GPC analysis method

Prepared concentration (ng/mL)	Measured concentration (ng/mL)	Recovery (%)
25	26.57±0.43	106.28±1.72
100	104.23±1.25	104.23±1.25
250	251.28±2.11	100.51±0.84
		Mean ± SD = 103.67±2.39

Although the method was linear up to 50 µg/mL concentration of Boc-DAB, to minimise wastage during reaction progress monitoring, samples were always diluted appropriately to keep the analysis sample concentrations close to 250 ng/mL to achieve the accurate results.

Precision

The coefficient of variance was determined from the relative standard deviation within six injections of two samples, which were 1.0% for 50 ng/mL and 1.1% for 200 ng/mL Boc-DAB standard solutions in ACN. The intraday variation was 1.6% and 0.7% and the interday variation was 1.5% and 1.6% at 50 and 200 ng/mL Boc-DAB standard solutions in ACN, respectively. All results were within the acceptable criteria for intra- and interday repeatability of RSD < 5%.

LOD and LOQ

Minimum detectable and quantifiable concentrations were calculated from the baseline noise level and then experimentally found to be 5 and 15 ng/mL, respectively.

Selectivity

The selective amine-derivatization procedure and fluorescence detection by FLD made this method very selective. First, fluorescamine only converts molecules with free primary amine into a fluorescent entity and second, FLD detects only fluorescence signals with a precise excitation-emission wavelength pair. From Fig. 8d it can be clearly demonstrated that the assay can separate the analyte of interest from all other impurities.

Conclusion

The current study successfully functionalised PLGA with DAB to produce PLGA-DAB. The functionalized PLGA can be conjugated with any carboxylic acid containing ligand and ultimately used for nanoparticle preparation for targeted drug delivery. The validated GPC method has been successfully used to confirm and quantify the polymer-amine complex as well as to monitor reaction progress. This method can be further adapted for quantification of other water-insoluble polymers with free primary amine group(s).

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