



## Method matters: Development of characterization techniques for branched and glucose-poly(lactide-co-glycolide) polymers



Justin Hadar<sup>a</sup>, Sarah Skidmore<sup>a</sup>, John Garner<sup>a</sup>, Haesun Park<sup>a</sup>, Kinam Park<sup>a,b,\*</sup>, Yan Wang<sup>c</sup>, Bin Qin<sup>c</sup>, Xiaohui (Jeff) Jiang<sup>c</sup>, Darby Kozak<sup>c</sup>

<sup>a</sup> Akina, Inc., 3495 Kent Avenue, Suite A200, West Lafayette, IN 47906, USA

<sup>b</sup> Purdue University, Biomedical Engineering and Pharmaceuticals, 206 S. Martin Jischke Drive, West Lafayette, IN 47907, USA

<sup>c</sup> Food and Drug Administration, Center for Drug Evaluation and Research, Office of Generic Drugs, 10903 New Hampshire Avenue, Silver Spring, MD 20993, USA

### ARTICLE INFO

#### Keywords:

Branched PLGA  
Glucose-PLGA  
Branching number  
L:G ratio  
Q1 sameness

### ABSTRACT

Defining the qualitative sameness of parenteral formulations comprised of poly(lactide-co-glycolide) (PLGA) requires assays of the relevant properties of polymer from each formulation. Gel-permeation chromatography with quaternary detection (GPC-4D) has been previously applied to other polymers, and the relevant mathematical parameters for their characterization are available; however, such parameters have not been described for branched PLGA polymers. Little information is available for the determination of glucose within glucose-PLGA (Glu-PLGA) branched polymers. This study describes the experimental methods of defining the mathematical parameters for characterization of branched PLGA polymers and the validation of these parameters using known branched-PLGA standards. The glucose, used as an initiator, was tracked through the synthesis of Glu-PLGA by both <sup>13</sup>C NMR and enzymatic analysis. The analytical determination of the relevant parameters defining Glu-PLGA, such as the branching number, and the presence of glucose, requires the use of appropriate procedures experimentally validated in a systematic manner. The procedures described in this study were developed for characterization of Glu-PLGA with the lactide:glycolide (L:G) ratio of 55:45 used in Sandostatin<sup>®</sup> LAR. The procedures can also be used for characterization of Glu-PLGAs made of different L:G ratios.

### 1. Introduction

The selection of the right drug delivery system mainly depends on the nature of the drug. While oral drug delivery formulations are most popular, not all drugs can be delivered by the oral route. Currently, oral delivery of macromolecules, including peptides, proteins, and nucleotides, is impractical, and they are mostly delivered by the parenteral route. For sustained delivery of peptides, injectable, long-acting formulations have been developed, such as microparticles, in situ forming gels, and solid implants, all of which are based on biodegradable poly(lactide-co-glycolide) (PLGA) polymers [1,2].

A wide array of methods can be applied to manufacturing injectable, long-acting PLGA formulations. The drug release properties from each formulation depend largely on the properties of drugs and the PLGA types, having different molecular weight, endcap, lactide:glycolide (L:G) ratio, and molecular structure [3,4]. The molecular structure of PLGAs used in most clinical products is linear, but one product, Sandostatin<sup>®</sup> LAR depot delivering octreotide, is made of a branched PLGA, also known as a star-PLGA, more specifically, a glucose

core with attached PLGA chains (Glu-PLGA) [5]. Only recently, however, the molecular structure of Glu-PLGA was characterized, including the number of branches for each glucose unit [6].

Developing characterization methods for such unusual molecular structure is important not only for the quality control of products, but also for establishing Q1/Q2 sameness during the development of generic products. PLGA is a unique group of polymers that behave differently, i.e., solubility in solvents, depending on the L:G ratio [7]. This makes characterization of Glu-PLGA difficult. The characterization method previously described for Glu-PLGA was for Sandostatin<sup>®</sup> LAR, having the L:G ratio of 55:45 [6]. This work, done by using gel permeation chromatography with quaternary detection (GPC-4D), enables characterization of the branching number of PLGAs used widely in the drug delivery field. Previously, such characterization was typically reserved for other polymers, such as polystyrenes, polyolefins, and very high molecular-weight polymers.

The parameters used in our previous work and this study are specific for the PLGA with the L:G ratio of 55:45, and other PLGAs with different L:G ratios will require reestablishing the necessary

\* Corresponding author at: Akina, Inc., 3495 Kent Avenue, Suite A200, West Lafayette, IN 47906, USA.

E-mail address: [kpark@purdue.edu](mailto:kpark@purdue.edu) (K. Park).

<https://doi.org/10.1016/j.jconrel.2020.02.005>

Received 29 November 2019; Received in revised form 19 January 2020; Accepted 2 February 2020

Available online 03 February 2020

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parameters. It is critical for a researcher to remember that GPC-4D is only a comparative technique that only provides relative information regarding the variation in occupied volumes between sets of polymers. The rest of the information gained from this technique (e.g., the degree of branching, size, dispersity of arms, etc.) is solely derived from the mathematical equations applied to the data. The parameters used in the equations must be determined experimentally and can only be validated by using polymers of the known branching status, which are not readily available for PLGA polymers. In this report, a detailed systematic process is described for experimental determination and validation of various parameters. This study presents additional, more detailed, information that can aid in analyzing the molecular structure of star-PLGA polymers having different L:G ratios.

## 2. Experimental methods

### 2.1. Materials

Glucose, Stannous octoate, toluene, trimethylolpropane, 1,2 hexadiol, pentaerythritol, adonitol, dipentaerythritol, dessicant packs (Aldrasorb), Glucose and Sucrose Assay Kit (Sigma MAK013-1KT) and activated charcoal were purchased from Sigma-Aldrich. Acetone (ACE), acetonitrile (ACN), dichloromethane (DCM), hexane, and tetrahydrofuran (THF) were purchased from Fisher Scientific. Ethanol (200 proof, ETOH) was obtained from Decon Laboratories, Inc. Glycolide and lactide were purchased from Ortec, Inc. and used as received.  $^{13}\text{C}$ -labelled glucose was purchased from Cambridge Isotopes. Glu-PLGA was kindly provided by Corbion. Additionally, PLGA polymers were purchased from Akina, Evonik, and Lactel. Sandostatin® LAR depot (30 mg) was obtained from WE Pharma (Morrisville, NC). Stannous octoate was vacuum distilled before usage. All other reagents were used as received unless otherwise specified.

### 2.2. Polymer synthesis

Since most of the polymer components in this study were not commercially available, they were synthesized in our laboratory, as described previously [6]. Briefly, an initiator for either a branched PLGA standard (trimethylolpropane, pentaerythritol, adonitol, and dipentaerythritol for 3, 4, 5, and 6 arms, respectively, and 1,2 hexadiol for 'bent' PLGA) or a Glu-PLGA (with  $^{13}\text{C}$ -labelled glucose) was dehydrated by azeotropic distillation from toluene followed by an argon flush. Afterwards, predetermined molar equivalents of lactide (L) and glycolide (G) monomers were added to the initiator along with the stannous octoate catalyst, and polymerization was initiated with heating 130–150 °C for approximately 8 h. Subsequently, the crude polymer was dissolved in DCM, filtered, and precipitated in hexane and washed with hexane and ethanol. It was then dried under vacuum at 50–60 °C until reaching a constant mass. Glu-PLGA was decolorized by dissolution in a 15 × volume of acetone and mixing with an equal mass of activated charcoal followed by filtration and vacuum drying. As a test for a glucose side-reaction, a 'faux' synthesis was setup wherein glucose was prepared, combined with stannous octoate, and heated according to the same reaction procedures as used to make the polymers; however, no monomers were added.

### 2.3. Hydrolysis of sample for determination of glucose presence

Approximately 25 mg of each sample was placed in a glass scintillation vial, and 10.0 ml of 0.1 M NaOH was added and capped tightly. Vials were then placed in an incubator shaker at 50 °C and 100 RPM for two months. After incubation, the liquid portion of the sample was decanted to a new vial to collect the hydrolysate free from the undegraded solid sample. The hydrolyzed sample was dried by rotary evaporation to remove the water from the sample. Samples were then analyzed by either NMR or a glucose assay kit [8].

### 2.3.1. Glucose side-reaction

The competing non-specific oxidation of glucose with or without the stannous octoate ( $\text{Sn}(\text{Oct})_2$ ) catalyst was tested by differential scanning calorimetry (DSC, TA Instruments Q2000 instrument) to determine more details regarding this reaction. The samples (~2–7 mg) were placed in aluminum pans (TA instruments), and a DSC run was performed under 50 ml/min argon chamber flush to prevent oxygen exposure. The DSC was ramped from 20 °C to 300 °C at 10 °C/min intervals. The DSC was done on glucose, stannous octoate, and glucose combined with stannous octoate samples, respectively.

## 2.4. Polymer characterization

### 2.4.1. Gel-permeation chromatography - external standard (GPC-ES)

GPC-ES was performed as previously described [6]. Briefly, this was done using a Breeze-2 system with the 1 ml/min THF mobile phase against three sequential GPC columns (Phenomenex, Agilent). The samples were injected at a concentration of 2 mg/ml (100  $\mu\text{L}$ ). Empower software was used to compare the samples to a series of Agilent Easical polystyrene calibration standards to obtain number average molecular weight ( $M_n$ ), weight average molecular weight ( $M_w$ ), and polydispersity.

### 2.4.2. Gel-permeation chromatography quaternary detection (GPC-4D)

The GPC-4D system was comprised of a Dawn Heleos II (MALLS) coupled to a Dynapro Nanostar DLS via an optical cable, Optilab T-REX (refractive index (RI) detector) and Viscostar III viscometer operated by Astra 7 software (Wyatt). The separation was performed with an Agilent 1260 Infinity II HPLC system using a linear gradient column (Tosoh Bioscience LLC, TSKgel GMH<sub>HR</sub>-L, 7.8 mm × 30 cm). Column compartment, viscometer, and RI detector were held at 25 °C.

This system was initially equipped with tetrahydrofuran (THF) as the mobile phase. It was primarily due to THF being a common solvent for polymer GPC. Additionally, acetonitrile (ACN) and acetone (ACE) were tested because of their improved performance compared to THF. Flow rates ranging from 0.3 to 1.0 ml/min were examined. The sensitivity of light scattering detectors was inversely related to the flow rate, while the viscometer response was directly proportional to the flow rate. It created a tradeoff between detector response when selecting the flow rate. Increased flow rates also provide the benefit of shortened run times.

### 2.4.3. Nuclear magnetic resonance (NMR)

NMR was performed as previously described [6]. Briefly, each sample was dissolved in 0.8 ml of an appropriate deuterated solvent (either chloroform ( $\text{CDCl}_3$ ) or methyl sulfoxide ( $\text{DMSO}-d_6$ )) in a 7 in × 5 mm NMR tube (Wilmad). These were then scanned by the PINMRF group at Purdue University on a Bruker AV-III-500-HD NMR spectrometer according to methods described previously [7,9,10]. NMR post-analysis was performed using an ACD/Specrus Processor (2015 Pack 2, ACD Labs). Proton counting on  $^1\text{H}$  NMR was used to determine the L:G ratio for all PLGA materials (lactide at 5.2 ppm for 1H; glycolide at 4.8 ppm for 2H). For branched-standard PLGA products, the degree of esterification was confirmed according to the peak shift of the branched initiator from 3.7 ppm (free) to 4.1 ppm (esterified).  $^{13}\text{C}$  NMR was used to inspect samples for peaks indicative of  $^{13}\text{C}$ -labelled glucose and to determine the blockiness ( $R_c$ ) according to the shift of the glycolide carbonyl from 166.3 ppm (adjacent to another glycolide) to 166.4 ppm (adjacent to another lactide).

## 2.5. Investigation of system parameters

Several system variables were examined to optimize data quality and accuracy. These included solvent selection for mobile phase, solvent  $dn/dc$ , sample concentration, and injection volume, and software parameters for data processing. THF, ACN, and ACE were tested for

possible mobile phase selections. Ultimately ACE was chosen for its chromatographic compatibility with PLGA polymers and its enhanced  $dn/dc$  properties. Solvent  $dn/dc$ , one of the critical input parameters for software calculations, was determined as previously described [6] by using batch mode analysis in Astra 7 software. Varying sample concentrations (2.5–5.0 mg/ml) and injection volumes (20–100  $\mu$ l) were examined for their effects on chromatography and detector response. Analysis of the branching number by the Astra 7 software required input of parameters, such as a method of determining branching (radius, molar mass, or viscosity), model (trifunctional, tetrafunctional, comb, or star), slice (monodisperse or polydisperse), and drainage factor.

### 3. Results

#### 3.1. PLGA standards

Both synthesized and commercially purchased PLGAs were characterized by NMR and GPC-ES. The properties of most of these polymers were detailed in Table 2 of our previous publication [6]. Linear PLGAs and Glu-PLGAs were obtained from Akina, Evonik, and Lactel. Branched PLGA standards with the branching number ranging from 2 (U-shaped) to 6 were synthesized.

#### 3.2. Synthesis of branched PLGA standards and determination of esterification

For branched PLGA polymers, the degree of esterification can be determined by NMR through monitoring the transition of the alpha-hydroxy hydrogens from 3.7 ppm (loose -OH moiety) to 4.1 ppm (esterified -OH moiety) [11]. Fig. 1 shows the determination of the degree of esterification of the multi-arm PLGA structure as examples. For the NMR analysis of degree of branching, the processing was performed using ACD/Spectrum 2015 on the region of interest between 2 and 5 ppm. For materials not included in the original publication [11], such as TMP-3-arm PLGA, the assignments were obtained from other literature sources. The peak assignments of TMP were obtained from Automated Topology Builder (ATB) and Repository [12]. The glycerol peaks were assigned using published triglyceride assignments [13]. Glycerol hydroxyl assignments were obtained from the Spectral Database for Organic Compounds SDBS [14].

The NMR method, however, cannot be used to determine the esterification of hydroxyl groups of glucose. The majority of glucose hydroxyl groups in the glucose ring structure lacks the alpha-hydrogens which shift upon transition from free to bound. Determining details regarding the glucose moiety post ring-opening polymerization is not readily achieved by conventional NMR and FTIR techniques. Thus, the branched PLGA standards with the branching number ranging from 3 to 6 were synthesized and verified, and the peak assignments of each branched PLGA are listed in Table 1. Table 1 shows the results of the degree of esterification calculated by the peak integration. The unreacted hydroxyl peak was below the detectable limit in practically all samples, indicating that the branched initiators were well esterified. The results in Table 1 indicate all hydroxyl groups were esterified, but it does not necessarily eliminate the possibility that linear PLGA may exist with the branched polymer.

#### 3.3. A side reaction of glucose molecules

The synthesis of Glu-PLGA is hindered by a side reaction of glucose which rapidly converts it to a dark brown/black color due to oxidation. This reaction was investigated by DSC (Fig. 2 A&B). Glucose has a minor dehydration peak at 75 °C followed by unspecified oxidation at 160 °C. Stannous octoate (by itself) has no notable transitions within this range. The combination of the two ingredients appears to lower the oxidation transition from 160 °C to 149 °C with onset at 129 °C, which is

well within the range of reaction temperatures typically applied for ring-opening polymerization. The noise after the peak in Fig. 2B is due to the simultaneous chemical decomposition (typically endothermic) and polymerization (typically exothermic) processes occurring simultaneously with the glucose at high temperatures. This effect was further investigated by performing a 'faux' reaction in which  $^{13}\text{C}(1,2)$ -glucose (0.1 g) and stannous octoate (0.17 g) were combined with no lactide or glycolide monomers and processed under the same reaction conditions (130 °C for 8 h) as that for synthesis of Glu-PLGA. The glucose was visibly observed to turn dark brown at the end of the reaction. The color formation also occurs during the synthesis of Glu-PLGA, and the color was subsequently removed by activated charcoal filtration.

The two components, stannous octoate and glucose, were tested by NMR individually before and after the faux-reaction (Fig. 3). Largely, the resultant NMR from the faux reaction resembles simply the addition of the glucose and  $\text{Sn}(\text{Oct})_2$  with minimal formation of new peaks. It indicates that although this side-reaction has a drastic effect on the appearance of the product, a relatively small fraction of the glucose participates in this reaction.

#### 3.4. Enzymatic measurement of glucose content

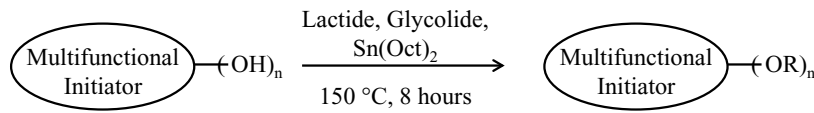
To establish Q1/Q2 sameness the identity of the PLGA used should be matched between a Reference Listed Drug (RLD) and the proposed generic formulation. Since the manufacturer of the RLD would have no reason to use  $^{13}\text{C}$ -labelled glucose for its formulation, a method of the glucose identification which does not rely on isotope labelling is necessary. Previously, the presence of glucose in synthesized Glu-PLGAs was confirmed by  $^{13}\text{C}$  NMR using  $^{13}\text{C}$ -labelled glucose after hydrolysis [6]. As explained, the use of  $^{13}\text{C}$ -labelled glucose, however, was not applicable for confirming the presence of glucose in a clinical product. In the absence of the  $^{13}\text{C}$ -label, the glucose peaks were not readily discerned among the peaks of other hydrolyzed products, such as lactic acid, glycolic acid, and oligomers. Fig. 4 shows resultant NMR data obtained from Glu-PLGA (Corbion). Similar NMR spectra were observed for Glu-PLGA from Evonik (L:G = 55:45,  $M_n$  = 40,178,  $M_w$  = 62,762, 5545 DLG 5GLU) and Akina (L:G = 45:55,  $M_n$  = 32,941,  $M_w$  = 55,320, AP029).

An enzymatic measurement was used to clarify further the presence of glucose in Glu-PLGA [16]. Hydrolyzed Glu-PLGA samples were tested using a glucose assay kit. Although this assay is designed to be used with a plate reader in 96 well plates, measurements were made with a UV-Vis spectrophotometer in microcuvettes. Erratic and non-linear results were obtained from the glucose standards. This seems to be a result of the exceedingly small volumes used and the poor light-path coverage of the microcuvettes. The enzymatic method, however, provided colorimetric proof that glucose exists in Glu-PLGA samples. Glu-PLGA samples presented an absorbance greater than the blank and visual color change to pinkish red as in the glucose standards. A linear PLGA was also put through the hydrolysis procedure, and its lack of absorbance and color change confirmed the absence of glucose. While this method as used did not provide a quantitative measurement of the glucose content, it confirmed the presence of glucose in Glu-PLGA.

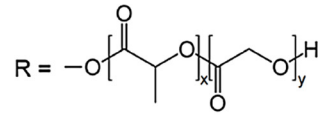
#### 3.5. Use of GPC-4D data for determination of the molecular structure of Glu-PLGA

Our previous study analyzed the branch units of Glu-PLGA of Sandostatin® LAR [6] using GPC-4D. The four detectors for refractive index, static light scattering, dynamic light scattering, and intrinsic viscosity do not directly provide the information on the branch units. Unless otherwise indicated, the mathematical modeling parameters used in this study are the same as described previously [6]. Interpretation and analysis was performed using Astra 7 software (Wyatt Technologies) and the equations utilized are detailed in the Astra 7 Users Guide (M1006 Rev.D).

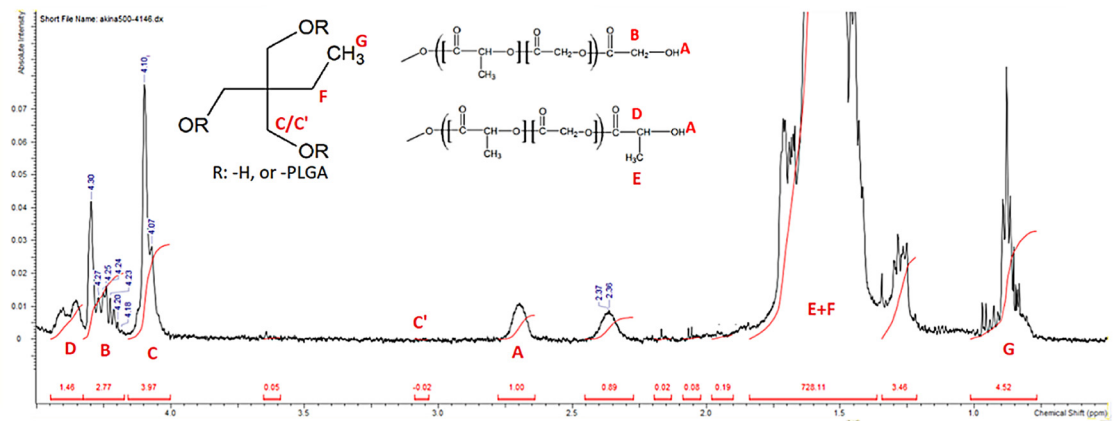
A



B



C



D

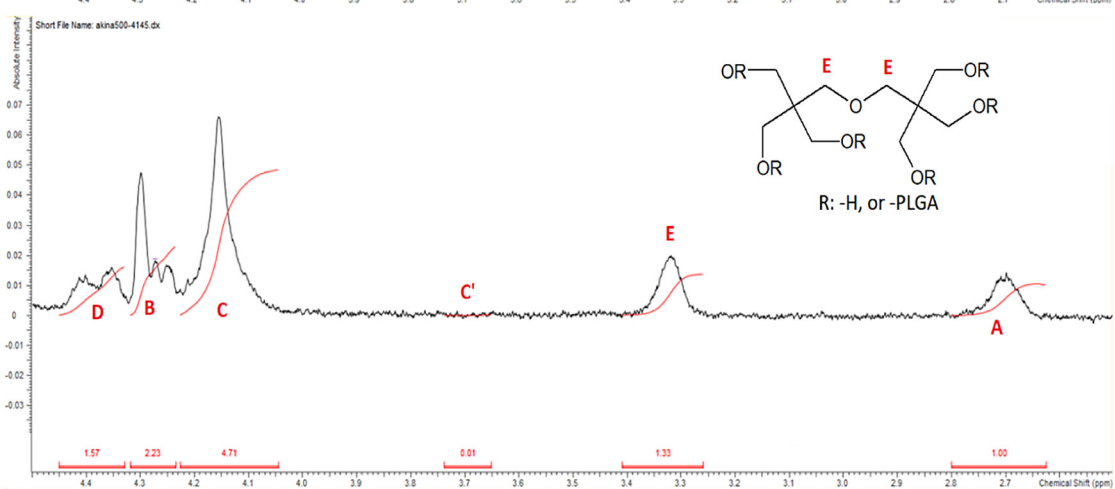
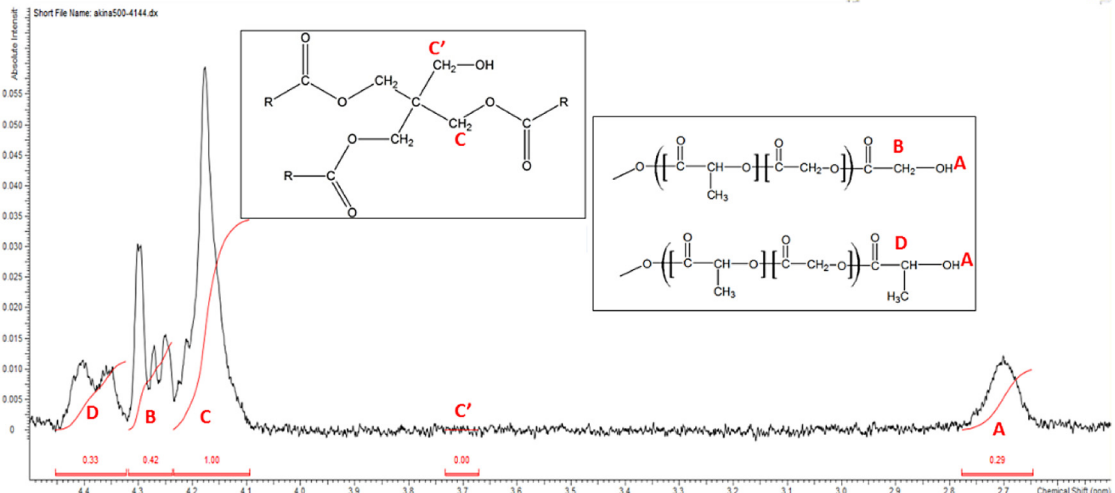


Fig. 1. (A) Generalized reaction schematic for formation of branched PLGAs. Peak assignments for determination of esterification by NMR of 3-arm PLGA (B), 4-arm PLGA (C), and 6-arm PLGA (D).

**Table 1**  
Peak integrations for calculation of the degree of esterification.

Polymer	Assignment	Location	Integration
PLGA-3 Arm (Akina AP237): L:G = 75:25, $M_n$ = 47,586, $M_w$ = 59,930	Hydroxyl peak	3.7 ppm	-0.03 <sup>a</sup>
	Ester peak	4.1 ppm	1.00
PLGA-3 Arm (Akina AP229): L:G = 56:44, $M_n$ = 44,029, $M_w$ = 57,854	Hydroxyl peak	3.2 ppm	-0.02 <sup>a</sup>
	Ester peak	4.1 ppm	3.97
PLGA-3 Arm (Akina AP237): L:G = 75:25, $M_n$ = 47,586, $M_w$ = 59,930	Hydroxyl peak	3.7 ppm	-0.03 <sup>a</sup>
	Ester peak	4.1 ppm	1.00
PLGA-4 Arm (Akina AP227): L:G = 57:43, $M_n$ = 47,129, $M_w$ = 59,686	Hydroxyl peak	3.7 ppm	0.00
	Ester peak	4.1 ppm	1.00
PLGA-5 Arm (Akina AP236): L:G = 53:47, $M_n$ = 46,028, $M_w$ = 57,126	Hydroxyl peak	3.7 ppm	0.00
	Ester peak	4.1 ppm	1.32
PLGA-5 Arm (Akina AP238): L:G = 77:23, $M_n$ = 53,800, $M_w$ = 65,607	Hydroxyl peak	3.7 ppm	-0.06 <sup>a</sup>
	Ester peak	4.1 ppm	2.29
PLGA-6 Arm (Akina AP228): L:G = 56:44, $M_n$ = 49,576, $M_w$ = 59,275	Hydroxyl peak	3.7 ppm	0.01
	Ester peak	4.1 ppm	4.71

<sup>a</sup> The negative signal indicates no observable peak.

### 3.5.1. Determination of solvent $dn/dc$

The refractive index response is linearly related to the sample concentration. The refractive index increment, or  $dn/dc$ , value refers to the change in the refractive index as a function of the PLGA concentration. This value is related to the difference in refractive index of the polymer and the solvent in which it is dissolved. It describes the degree to which polymer solutions will refract (bend) light as compared to the neat solvent. The  $dn/dc$  parameter is critical in interpretation of the resultant data as the concentration determined by the refractive index detector relies on the  $dn/dc$ . It works the same way as a UV-Vis instrument measures the concentration based on a compound's molar absorptivity. Furthermore, light-scattering determination of a polymer molecular weight by the MALLS system is reliant on the polymer's  $dn/dc$  which is a part of the universal calibration equations.

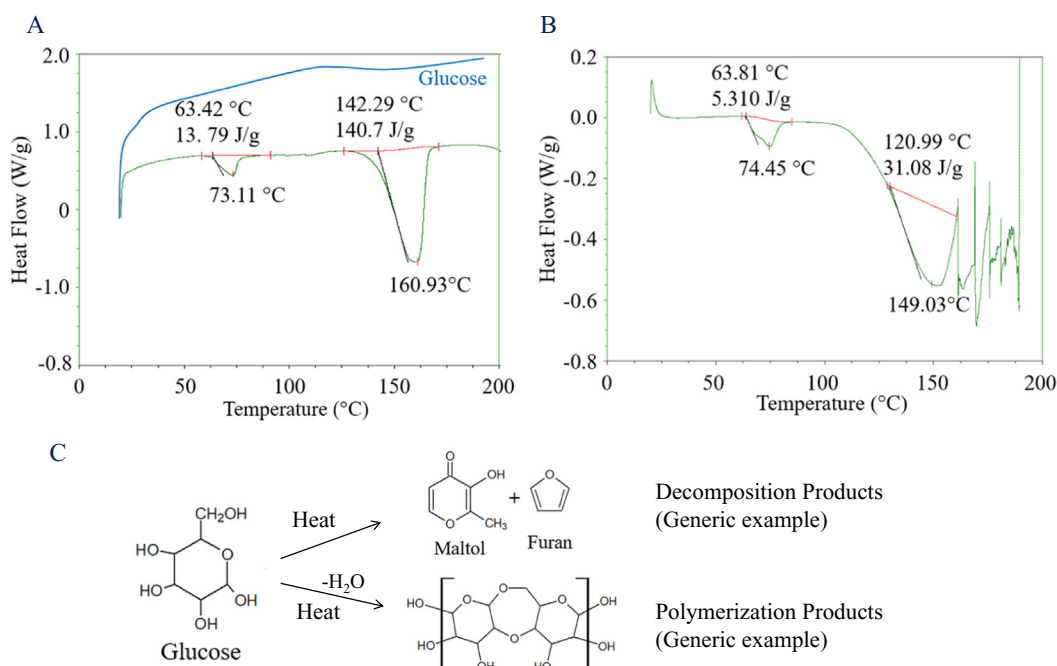
The  $dn/dc$  of PLGA depends on the L:G ratio, and the  $dn/dc$  obtained for PLGA of a specific L:G ratio cannot be used for other PLGAs having different L:G ratios [17]. Table 2 shows determined  $dn/dc$  values for PLGAs with different L:G ratios and molecular structures. As the data of linear PLGAs show, the  $dn/dc$  value increases as the L:G ratio decreases

from 100:0 to 50:50. Thus, entering the  $dn/dc$  value of PLGA 75:25 for PLGA 55:45, for example, results in wrong information. At a given L:G ratio, the  $dn/dc$  value appears to increase, although slightly, as the molecular weight increases. The  $dn/dc$  value also increases for branched PLGAs, i.e., more compact molecular structures. The importance here is that the  $dn/dc$  value needs to be measured for each PLGA to obtain an accurate understanding of the molecular structure of PLGA from GPC-4D data.

For GPC-4D experiments, the  $dn/dc$  value, which is one of the required inputs, is critical for the RI detector to determine the PLGA concentration. Thus,  $dn/dc$  for an unknown sample should be determined carefully for obtaining accurate data from GPC-4D [18].

### 3.5.2. Determination of solvent effect on data quality

One of the most common mobile phases used for GPC is THF. It dissolves a broad range of polymers and is readily available. For this reason, THF was used initially in our study. It was noticed, however, that THF is not the best solvent for PLGA. The refractive indices of both THF and PLGA are fairly similar, and the  $dn/dc$  is typically very low in



**Fig. 2.** DSC analysis of  $\text{Sn}(\text{Oct})_2$  (A) and  $\text{Sn}(\text{Oct})_2$  + glucose (B). The glucose DSC is shown in blue in A. (C) Generic schematic of oxidation of glucose to form decomposition products and polymerized products [15]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

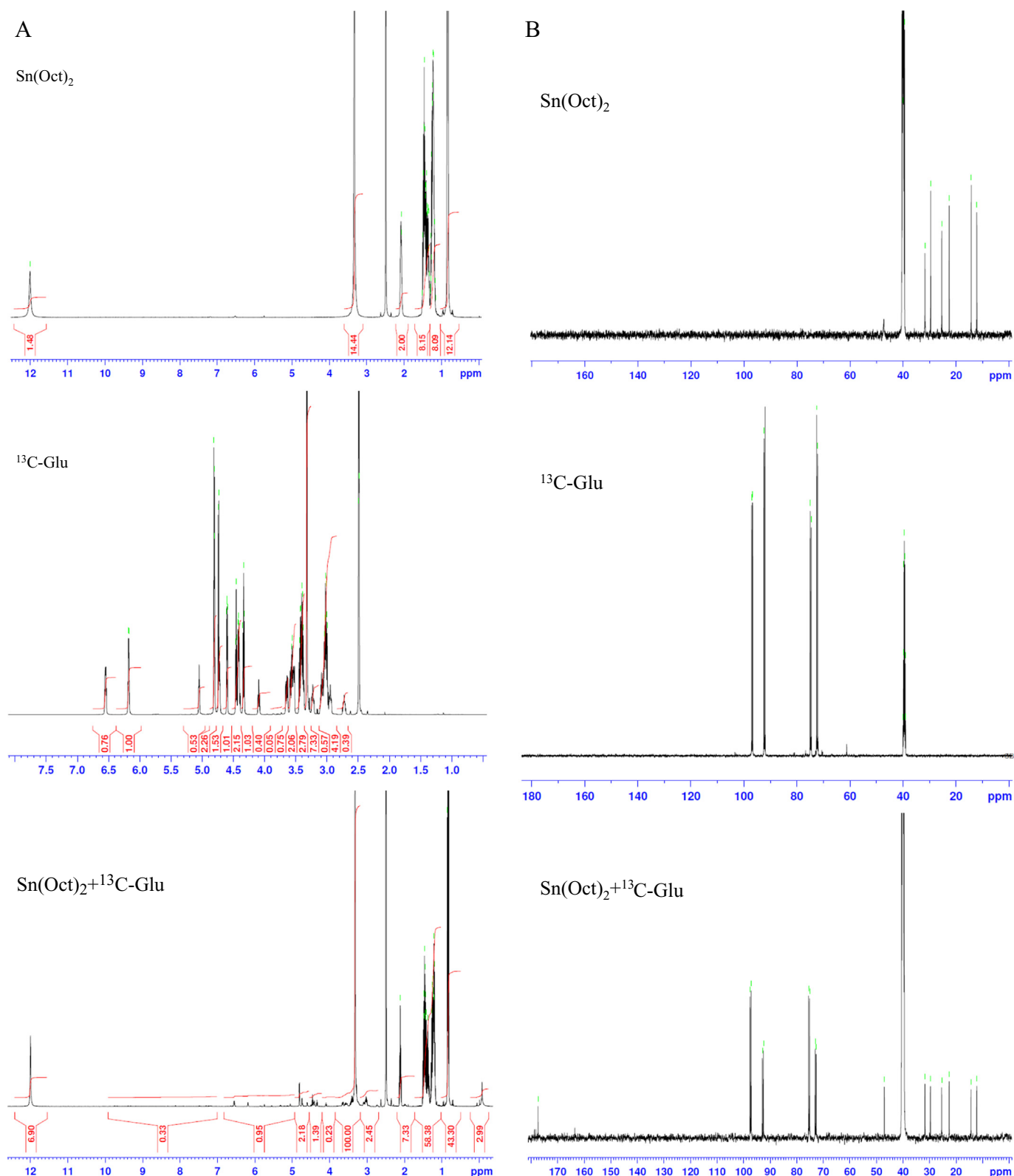


Fig. 3. <sup>1</sup>H NMR (A) and <sup>13</sup>C NMR (B) analysis of Sn(Oct)<sub>2</sub> and <sup>13</sup>C-labelled (C1,2)-glucose (<sup>13</sup>C-Glu) before and after faux-reaction (Sn(Oct)<sub>2</sub> + <sup>13</sup>C-Glu). NMR was performed in DMSO-d<sub>6</sub>.

the range of 0.04–0.06 [19]. THF has a refractive index of 1.407 at 20 °C [20]. On the other hand, acetone, with a refractive index of 1.359 at 20 °C [21], presents a more pronounced *dn/dc* of 0.09–0.10 with PLGA [19]. The larger *dn/dc* of acetone made it easier to study PLGA with GPC-4D. Table 3 and Fig. 5 show the results from dynamic light

scattering (DLS) determination of hydrodynamic radius (*R<sub>h</sub>*) in both THF and acetone. For acetone, different PLGA loading conditions were examined.

As the data demonstrate, the DLS decay correlation and resulting data quality (% instrumental uncertainty) are drastically improved

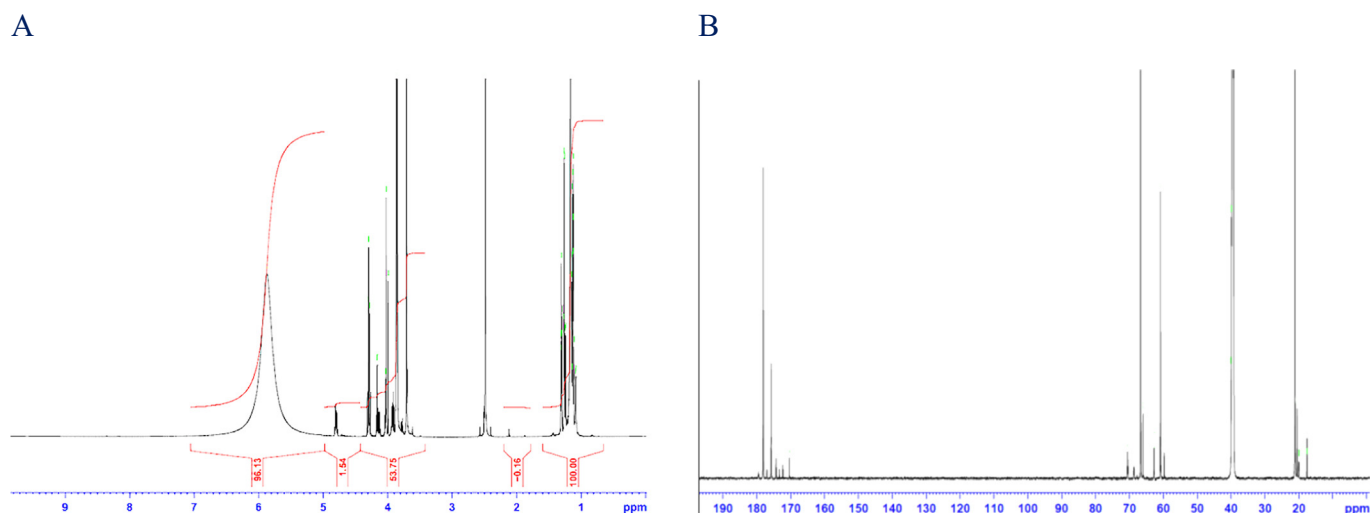


Fig. 4.  $^1\text{H}$  NMR (A) and  $^{13}\text{C}$  NMR (B) analysis of Glu-PLGA after hydrolysis. Glu-PLGA was obtained from Corbion (L:G = 55:45,  $M_n = 44,834$ ,  $M_w = 69,351$ , Purasorb PDLG5505G). Glucose was not labelled with  $^{13}\text{C}$ .

when acetone is used as the mobile phase, instead of THF. THF is a perfectly suitable mobile phase as the difference in  $dn/dc$  is sufficient for a conventional RI detector to observe the presence of the polymer. However, for GPC-4D applications and light-scattering methods, THF does not provide adequate data for accurate analysis, and acetone or other low-refractive index solvents provide significant advantages for light scattering analysis. As the concentration of PLGA increases from 125  $\mu\text{g}$  to 500  $\mu\text{g}$ , the signal becomes substantially higher for a more accurate analysis of the molecular weight and molecular structure of PLGAs.

### 3.5.3. Effect of the selection of linear PLGAs

The selection of the PLGA type used as the linear comparator is critical for the accurate determination of branch units of Glu-PLGA. A calculation of the branch units per molecule requires a relevant linear PLGA standard against which this data is compared. The linear standards need to have identical solvation properties and a similar molar mass to the branched polymers being tested. Since the solvent solubility of PLGA depends on the L:G ratio, linear PLGAs need to have the same L:G ratio with the branched PLGA being tested. To show the impact of using linear PLGAs with different L:G ratios on the calculated branch units, linear PLGAs with three different L:G ratios (50:50, 55:45, and 75:25) were used as linear comparators for a PLGA-5 arm.

Fig. 6 shows the results of using three different L:G ratios of PLGAs with similar molecular weights. The calculated branch units per molecule are drastically different simply using linear PLGAs of different L:G ratios. It is noted that the calculated branch units are substantially different by simply changing the L:G ratio from 55:45 to 50:50. Only

the linear PLGA with 55:45, which is the same as 5-arm PLGA, resulted in an accurate calculation of the branch units. The deviation from the nominal branching value increases as the difference in the L:G ratio between the linear and branched PLGAs widens. This data was collected using a drainage factor of 0.805, which was selected by testing different drainage factors. The drainage factor is a software input for data interpretation which relates to how the polymer interacts with the solvent and must be experimentally determined.

5-arm PLGA (L:G = 53:47,  $M_n = 46,028$ ,  $M_w = 57,126$ , Akina AP236);

linear PLGA 55:45 (L:G = 55:45,  $M_n = 36,112$ ,  $M_w = 51,921$ , Akina AP076);

linear PLGA 50:50 (L:G = 50:50,  $M_n = 40,763$ ,  $M_w = 60,889$ , Evonik RG504H); and.

linear PLGA 75:25 (L:G = 75:25,  $M_n = 51,168$ ,  $M_w = 72,176$ , Evonik R755S).

This solvation effect is so critical that even the endcap can have an impact on the apparent branch unit calculation. When one linear polymer is compared to itself (Fig. 7, green, acid endcapped) for calculation of branch units, the result is a flat line with two branch units per molecule across the molar mass distribution. However, another linear PLGA with an ester endcap was examined, the branch units per molecule were not quite 2. The branch units rose up to 2.6. This indicates that even slight differences in the PLGA properties may affect the calculation of the branch units per molecule.

Green: Acid endcapped linear PLGA (L:G = 55:45,  $M_n = 36,112$ ,  $M_w = 51,921$ , Akina AP076);

Red: Ester endcapped linear PLGA (L:G = 54:46,  $M_n = 22,779$ ,

**Table 2**  
PLGA  $dn/dc$  values measured in acetone.

Polymer	Determined $dn/dc$
Linear PLGA (Akina AP071): L:G = 100:0, $M_n = 38,513$ , $M_w = 57,374$	$0.0911 \pm 0.0015$
Linear PLGA (Evonik R205S): L:G = 100:0, $M_n = 53,352$ , $M_w = 76,949$	$0.0917 \pm 0.0004$
Linear PLGA (Evonik R858S): L:G = 85:15, $M_n = 122,496$ , $M_w = 179,454$	$0.0930 \pm 0.0014$
Linear PLGA (Evonik R755S): L:G = 75:25, $M_n = 51,168$ , $M_w = 72,176$	$0.0950 \pm 0.0010$
Linear PLGA (Evonik RG653H): L:G = 65:35, $M_n = 22,400$ , $M_w = 34,944$	$0.0968 \pm 0.0008$
Linear PLGA (Evonik RG504H): L:G = 50:50, $M_n = 40,763$ , $M_w = 60,889$	$0.0977 \pm 0.0014$
Glu-PLGA (Evonik 5545 5GLU): L:G = 55:45, $M_n = 40,178$ , $M_w = 62,762$	$0.0987 \pm 0.0012$
Glu-PLGA (Corbion G5505G): L:G = 55:45, $M_n = 44,834$ , $M_w = 69,351$	$0.0996 \pm 0.0026$
3-Arm PLGA (Akina AP229): L:G = 56:44, $M_n = 44,029$ , $M_w = 57,854$	$0.0973 \pm 0.0007$
4-Arm PLGA (Akina AP227): L:G = 57:43, $M_n = 47,129$ , $M_w = 59,686$	$0.0980 \pm 0.0007$
6-Arm PLGA (Akina AP228): L:G = 56:44, $M_n = 49,576$ , $M_w = 59,275$	$0.0980 \pm 0.0012$

**Table 3**

Determination of hydrodynamic radius ( $R_h$ ) of PLGA-4 arm (L:G = 57:43,  $M_n = 47,129$ ,  $M_w = 59,686$ , Akina AP227) by DLS. (Flow rate of 0.6 ml/min).

Sample	PLGA Load	Mobile phase	$R_h$ (at $M_n$ ) (nm)	$R_h$ (at $M_w$ )
A	250 $\mu$ g	THF	2.476 ( $\pm$ 83.4%) <sup>a</sup>	2.643 ( $\pm$ 83.1%)
B	125 $\mu$ g	Acetone	4.969 ( $\pm$ 52.9%)	5.159 ( $\pm$ 51.1%)
C	250 $\mu$ g	Acetone	5.049 ( $\pm$ 30.4%)	5.350 ( $\pm$ 28.3%)
D	500 $\mu$ g	Acetone	5.185 ( $\pm$ 19.1%)	5.503 ( $\pm$ 16.7%)

<sup>a</sup> The % values inside parentheses are instrumental uncertainty values.

$M_w = 32,595$ , Akina AP063);

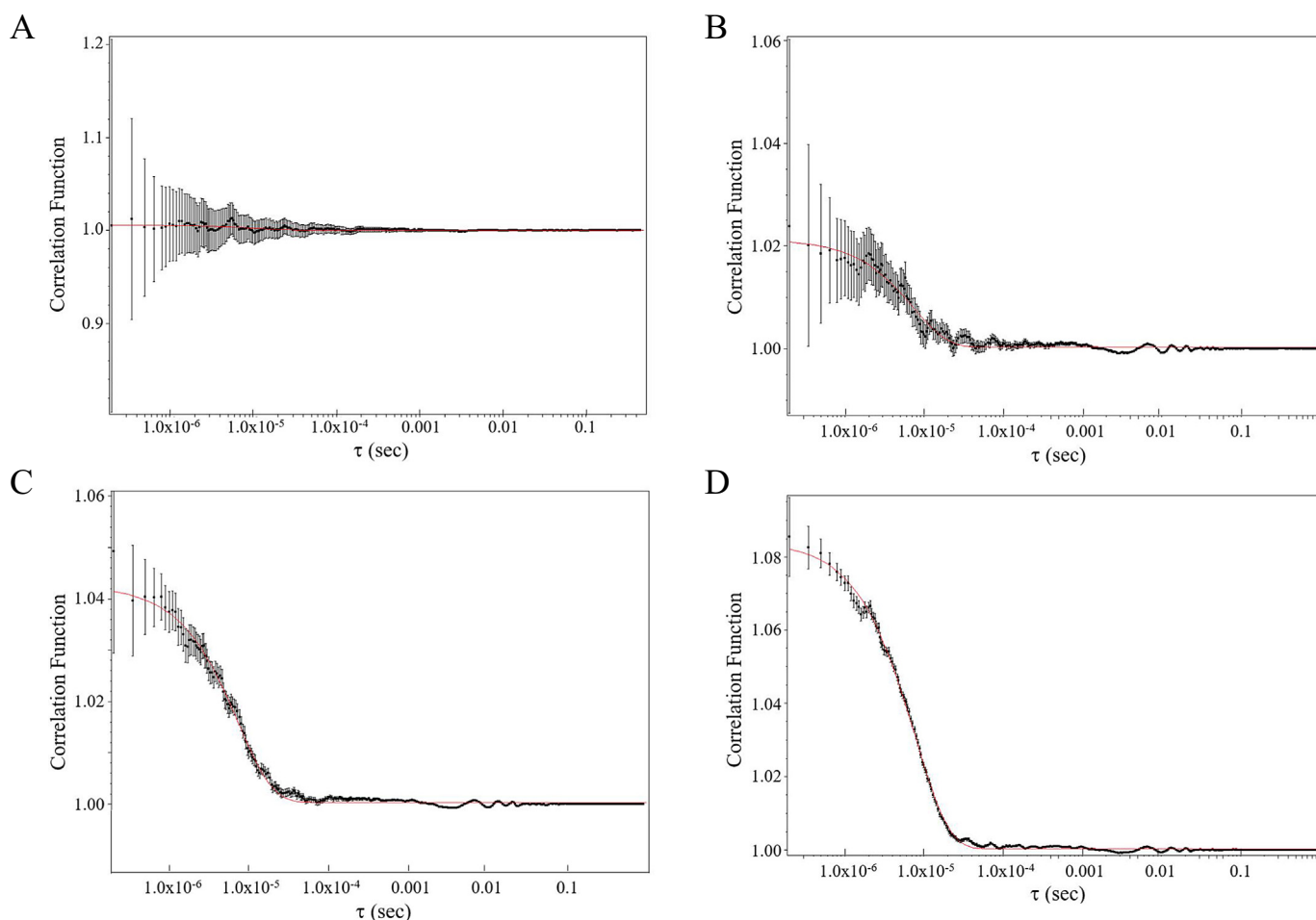
### 3.5.4. Molecular weight

The type of linear PLGA comparators had a large impact on the calculation of branch units. The L:G ratio and molar mass are both important. It was especially true at the upper end of the molar mass. Initially, a linear PLGA was chosen to have the same L:G ratio and similar molar mass (L:G = 55:45,  $M_n = 36,112$ ,  $M_w = 51,921$ , Akina AP076) as the branched standards. The branch unit values tended to decrease at the higher molar mass range. This was counter to the expectation that branching would increase with increasing molar mass. Further examination revealed that the linear comparison chosen did not cover the full range of molar masses present in the branched polymers being examined. This is one crucial aspect of the linear comparison. The Astra 7 software must have comparison values at the greatest overlap of molar masses to calculate branch units per molecule properly. Subsequently, another linear PLGA comparator was chosen

(L:G = 55:45,  $M_n = 90,327$ ,  $M_w = 142,190$ , Akina AP036) to provide molar masses in the higher range. This higher molar mass linear comparator increased the range of molar masses that Astra 7 was able to calculate branching. However, this created an upward deviation in the branching at the lower end of molar mass, and eliminated the branch units below a certain molar mass. This deviation in the branch units was most impactful in the  $M_n$  and  $M_w$ . Since the largest population of molecules falls in this range, this would cause inaccurate obtained branch values. To compensate these effects and to obtain the most accurate branching data, a combination of the above two linear PLGAs was used. This allowed the molar mass overlap between the branched polymer and linear comparator. This effect is shown in Fig. 8 for 3, 4, 5, and 6-arm branched PLGA standards (PLGAs AP229, AP227, AP236, and AP228 in Table 1). It was also necessary to optimize the drainage parameter for using the high molar mass and mixed molar mass linear comparator. A final optimized drainage parameter of 0.750 was determined using the mixed linear comparison (Table 4, in bold). It is noted that the drainage factor of 0.805 was used in Fig. 6. The optimum drainage factor needs to be determined in each experiment using the known branched PLGA standards.

## 4. Discussion

Ring-opening polymerization of lactide and glycolide monomers is initiated by hydroxyl moieties in the presence of an appropriate catalyst, such as stannous octoate [22]. Glucose, a monosaccharide with five available hydroxyl sites when in ring form, can potentially initiate



**Fig. 5.** DLS decay correlations for PLGA-4 arm (L:G = 57:43,  $M_n = 47,129$ ,  $M_w = 59,686$ , Akina AP227) at 250  $\mu$ g in THF (A), 125  $\mu$ g in acetone (B), 250  $\mu$ g in acetone (C), and 500  $\mu$ g in acetone (D).



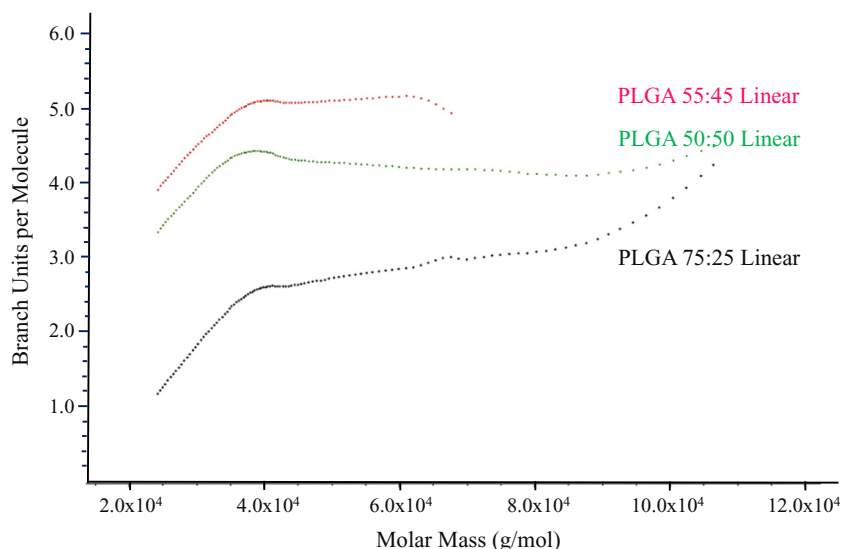


Fig. 6. Calculated branch units of 5-arm PLGA when using linear standards with the L:G ratio of 55:45 (red), 50:50 (green), and 75:25 (black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

up to five PLGA chains and occupy a site within the core of the polymer. However, these reactions often take place at temperatures above 130 °C where non-specific oxidation of the glucose molecule by processes similar to Maillard browning [23] can occur. Visually, this competing reaction is quite apparent, because the synthesis of Glu-PLGA by simple heat-initiated ring-opening polymerization yields a product which appears black/dark-brown in color due to the caramelizing side-reaction of glucose. Glucose is visibly destroyed under the conditions used for synthesis of Glu-PLGA. Thus, a question arises whether all glucose is destroyed, and if not, how much glucose survives the polymerization reaction condition. Since the Glu-PLGA used in Sandostat<sup>®</sup> LAR does not have <sup>13</sup>C-labeled glucose, it was difficult to quantify the glucose content, but the enzymatic analysis confirmed the presence of glucose in the sample product.

There remains a future potential to apply a small volume system, such as a microplate reader, to enable quantification of glucose according to the enzymatic method. This would correlate to the content of glucose within the hydrolyzant after separation from any remaining solid content. In order to be meaningful, this value must be correlated to the mass content of glucose in the original polymer. Since the actual degree of hydrolysis is unknown, this requires an assumption that the entire PLGA portion of the polymer was fully hydrolyzed with no damage occurring to the glucose during this process. Such an assumption may not be reasonable given the practical limitations of the PLGA

hydrolysis and potential for side reactions (such as non-specific oxidation) involving glucose. Due to these practical limitations, it is generally held that this method should be applied for qualitative evidence of glucose presence rather than exact quantification.

Despite extensive applications during the last several decades, PLGA polymers are still unfamiliar to us. Our understanding of PLGA, in general, is still limited. Until recently, it was difficult to separate different PLGAs if two or more different PLGAs were used in a formulation, such as in Trelstar<sup>®</sup> [7]. Furthermore, no assay methods were available to determine the accurate molecular structure of Glu-PLGA used in Sandostat<sup>®</sup> LAR. Notably lacking has been an analytical technique to determine the molecular structural information, such as linear or star-shaped, the number of arms, the dispersity of the arms, and other parameters [6]. These parameters are conventionally determined by the use of GPC-4D, including multi-angle laser light scattering (MALLS) [24], low-angle light scattering [25], and viscometers [26]. In each case, the detector is coupled to refractive index to determine the concentration of the polymer in the eluent. These techniques have been used previously for other polymers such as poly(vinyl acetate) [25], polyolefins [26], poly(methyl methacrylate), and poly(styrene) [24], but their use for PLGA has been limited.

The primary principle behind the branch analysis is that the chain extension of a branched polymer in a solvent is smaller as compared with the linear polymer counterpart [27]. Thus, the branching analysis

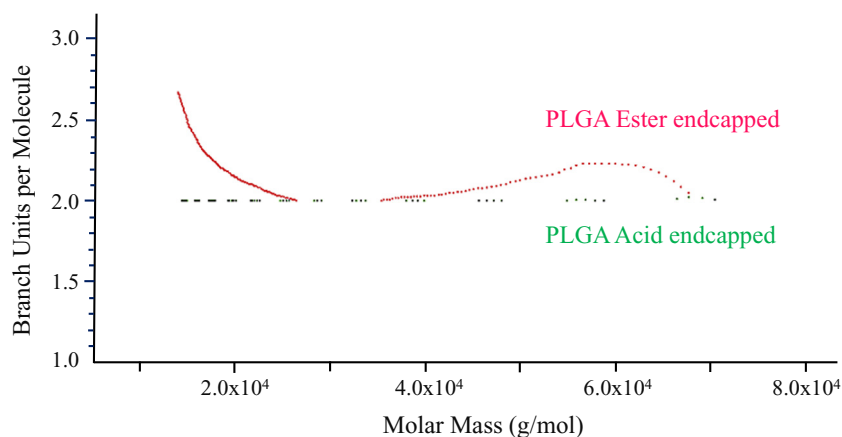


Fig. 7. The effect of PLGA endcaps on the calculation of branch units.

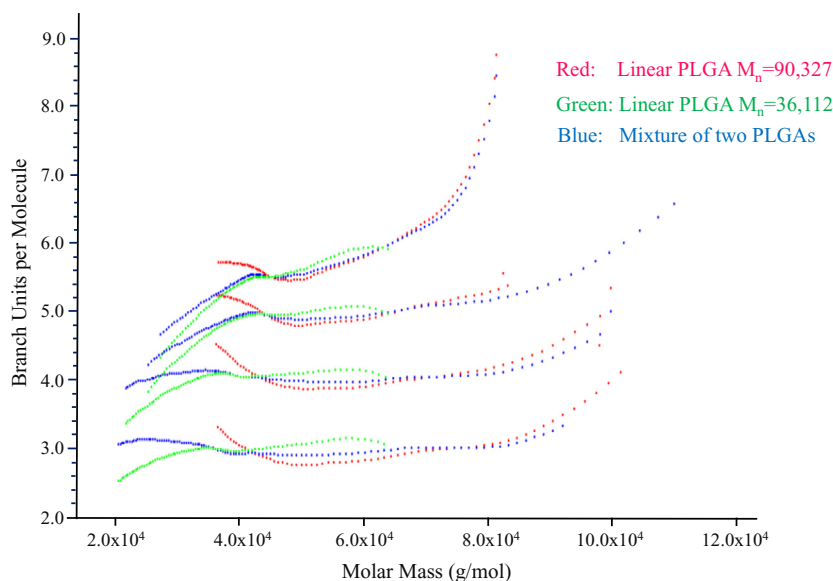


Fig. 8. Determination of branch units based on linear PLGA comparators having different molar masses.

Table 4

Selection of the optimum drainage factor by comparing calculated branch units per molecule at  $M_n$  for each branched PLGA standard and differences from nominal using a mixed molar mass linear comparator.

Drainage Factor	Branch Units Result				Sum of differences <sup>a</sup>
	3-Arm PLGA	4-Arm PLGA	5-Arm PLGA	6-Arm PLGA	
0.700	3.06	4.32	5.30	5.97	0.71
<b>0.750</b>	<b>3.00</b>	<b>4.17</b>	<b>5.07</b>	<b>5.69</b>	<b>0.55</b>
0.775	2.97	4.10	4.97	5.56	0.60
0.805	2.94	4.03	4.86	5.42	0.81
0.850	2.89	3.92	4.71	5.23	1.25

<sup>a</sup>  $\Sigma$  absolute(nominal – determined branching).

by GPC-4D relies on comparing the solvated radius of the branched polymer with that of a linear comparator of the same overall molecular weight [28]. Since these polymers are polydisperse, the use of GPC to separate specific molecular fractions according to their retention time is necessary to achieve uniformly dispersed fractions for comparison. Accordingly, light-scattering and viscometric techniques are applied to enable comparisons of the relative volume occupied by the polymer chains in a dilute solution.

GPC-4D methods have been used since the 1970s, but they have not been applied for the characterization of PLGAs, especially those used in clinical formulations. There are several reasons for this. The most widely used solvents in GPC, such as THF [29], have a refractive index that is very similar to that of PLGA, resulting in a very weak light-scattering signal [19]. Additionally, the PLGAs used in depot formulations are typically low molecular weights (< 100 kDa). Thus, they often exhibit a radius below 10 nm, and this presents practical limitations to static-light scattering approaches. They typically provide more robust data for molecules larger than 10 nm due to diffraction limitations [30]. Conventionally, branching assays by GPC-multidetector techniques have been restricted to fairly large molecules with strong light-interaction properties (dn/dc). Advances in the detector instrument have enabled finer-resolution measurements, and the use of lower refractive-index solvents has made it possible to determine the branching number in PLGAs with a molecular weight less than 100 kDa.

GPC-4D is a comparative technique that only provides relative information regarding the variation in occupied volumes between sets of polymers. The rest of the information gained from this technique (e.g.,

degree of branching, size, and dispersity of arms) is solely derived from the mathematical equations applied to this data. The constants and parameters to determine these equations must be determined experimentally and can only be validated by using standard polymers of known branch units. The branched PLGA standards were not readily available, and thus they were synthesized in our study and used for the analysis of Glu-PLGA in the Sandostatin® LAR formulations. Although the parameters established in this work only apply to PLGA 55:45 found in the Glu-PLGA used in Sandostatin® LAR formulation, the overall methodology to define these parameters can be broadly applied to PLGAs with different L:G ratios and other polymers.

Ring-opening polymerization is a relatively non-specific reaction. An appropriately activated hydroxyl (in this case, one in which the tin atom has attached to the oxygen) can activate a dimerized monomer to open initiating the polymerization process. The initiation of polymerization relies on activation of the hydroxyl group and its proximity to a dimer molecule. Thus, steric hindrance and other conditions can play a role in the synthesis of branched structures, as well as potential for competing side reactions. The side reactions can be prevented, if the core initiator is simple in structure. Glucose is not a good initiator for the synthesis of branched PLGAs, as it possesses other chemical moieties and readily undergoes a side-reaction (e.g., browning) during the polymerization reaction. For this reason, a series of hydroxy-alkyl initiators were used in this study.

Accurate determination of the branch units per Glu-PLGA molecule requires branched PLGA standards, which were synthesized in our laboratory. The branched PLGA standards with 3, 4, 5, and 6 arms made it possible to determine the PLGA branch units of Glu-PLGA of the Sandostatin® LAR formulations. Our study shows that accurate calculation of the branch units depends on the selection of the proper linear PLGA comparators that cover the entire molecular range of the branched PLGAs. More importantly, it is critical to use the PLGA of the same L:G ratio and endcap. The L:G ratio is probably the most influential factor in determining the optimal drainage factor that has the most impact on the calculation of the branch units.

## 5. Conclusion

Accurate analysis of PLGA properties requires the precise method for the assay. Analysis of Glu-PLGA is a good example demonstrating the need for the preciseness of assay methods. There has been little information in characterizing the molecular structure of Glu-PLGA. The

core-first synthesis of Glu-PLGA resulted in damages to the initiator during the polymerization reaction by a competing side-reaction of non-specific oxidation. It raised a doubt whether glucose survives the polymerization reaction. NMR measurements of Glu-PLGA provided little information on the presence of the glucose core, even after hydrolysis of the PLGA chains. However, the enzymatic assay of the  $^{13}\text{C}$ -labelled glucose-PLGA confirmed that the majority of glucose survived the reaction. The GPC-4D analysis showed that every detail is critical in obtaining accurate results for branching. It is imperative to use a comparable standard, appropriate loading concentration, an appropriate solvent, and an appropriate flow rate. They provide instrumental measurement data that enable the correct determination of branch units per molecule. Inputting the right  $dn/dc$ , drainage factor, and other experimentally determined variables is necessary for calculation of accurate branching information. Thus, development of analytical assays requires careful selection of branch standards and a systematic approach for validation.

### Acknowledgements

This work was supported by BAA Contract #HHSF223201710123C from the U.S. Food and Drug Administration (FDA). The content is solely the responsibility of the authors and does not necessarily represent the official views of the FDA. We would like to acknowledge Dr. John Harwood, Director Purdue Interdepartmental NMR Facility, for his contributions to this work.

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