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Amino Acid and Poly(Ethylene Glycol) Based Self-Organizing Polymeric Systems: Chemo-Enzymatic Synthesis and Characterization

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A chemo/regio selective enzymatic methodology has been designed to synthesize amphiphilic copolymers based on amino acid diesters and poly(ethylene glycol) [PEG]. The condensation polymerization was catalyzed by immobilized *Candida antarctica* lipase B (Novozyme 435) under solvent-less conditions. The synthesized polymers **3a–c** were derivatized with long chain acid chlorides by chemical acylation to get the amphiphilic polymers **4a–c**. The physical properties of the synthesized amphiphilic polymers *viz*: aggregation number, critical micelle concentration (CMC), radius of gyration (R_g), hydrodynamic radius (R_h) and particle size distribution were studied by static and dynamic light scattering (SLS and DLS) techniques. The polymers were found to be promising in drug delivery applications.

Keywords: Amphiphilic copolymer, Novozyme-435, *Candida antarctica* lipase, micelle, chemo/regioselectivity

1. Introduction

Polymers that are made of naturally occurring building blocks are preferable materials for biomedical applications as their degradation products are nontoxic and can be metabolized easily by living tissues. The immunogenicity of amino acid derived polymers are strongly dependent on their structures and conformations. Therefore, it is expected that hetero chain polymers having a backbone of appropriately chosen amino acids backbone would show lower immunogenicity than conventional poly (α-amino acids) (1, 2).

Polyethylene glycol (PEG) is one of the commonly used polymers or a part of copolymers in various drug formulations due to its outstanding physico-chemical and biological properties. These properties include hydrophilicity, solubility in water and in organic solvents, lack of toxicity, absence of antigenicity and immunogenicity which makes it one of the more valuable components for biocompatible materials (3). Various strategies have been reported in the literature utilizing PEG as one of the components to generate new classes of promising biomaterials. Most of these strategies often involve organometallic catalysts for generating these materials, requiring additional processing to remove the trace amounts of organometallic catalyst (4).

In the literature, some amino acid and poly(ethylene glycol) di and tri block copolymers are reported with a tedious protection and deprotection approach using hazardous chemicals, catalysts and harsh reaction conditions in their synthesis (4). In recent years polymeric micelles prepared from poly(ethyleneoxide–co-benzyl-l-aspartate) block copolymer (5) and their derivatives have received much attention as powerful drug carriers. Indeed, it has been shown that such polymeric micelles are stable in aqueous medium which solubilize hydrophobic drugs in their inner core (6, 7).

Growing awareness of the use of environmentally friendly processes have led us to investigate the enzyme catalyzed polymerization of PEG with various diesters of amino acids, to make the process and the resulting polymers highly biocompatible and environmentally friendly. This is in continuation of our earlier work on the synthesis of novel amphiphilic polymers for drug delivery applications (8–14). Herein, we report the synthesis of the novel amphiphilic polymers based on PEG and amino acid diesters catalyzed

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by Novozyme 435 and their post-functionalization to generate amphiphilic copolymers. The enzyme catalyzed co-polymerization was highly *chemo/regio* selective, and synthesis of such copolymers by conventional methods would have required additional protection and deprotection chemistry not to mention the use of metal catalysts.

2. Experimental

2.1. *Materials*

Novozyme-435, an immobilized enzyme, was a gift from Novozyme Inc., Denmark. Polyethylene glycol was dried under vacuum for 12 h before use and the monomers, diethylamino malonate hydrochloride, L-aspartic acid dimethyl ester hydrochloride, L-glutamic acid diethyl ester hydrochloride and poly(ethylene glycol) were purchased from Aldrich and used as received.

2.2. *Characterization*

Gel permeation chromatography (GPC) was used to determine the molecular weights and molecular weight distributions, Mw/Mn of polymer samples. The ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker DPX 200 spectrometer at 200 MHz and 50 MHz, respectively. Static light scattering data was collected on a laser light scattering photometer (Wyatt Technology DAWN Model) equipped with a 632 nm He-Ne Laser as a light source. Dynamic light scattering was performed using a 50 mW He-Ne Laser, an available photodiode detector (BI-APD), a digital time correlator (BI-9000) and software from Brookhaven Instruments Corporation.

2.3. *General procedure for neutralizing hydrochloride salt of amino hydrochloride diesters 1a–c*

To an ice cooled suspension of **1a–c** (0.01 mol) in anhydrous dichloromethane (30 ml), triethyl amine (0.01 mol) was added dropwise with constant stirring. The reaction mixture was left at room temperature for an hour with stirring. After completion, the reaction mixture was poured onto crushed ice, and extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulphate, filtered and concentrated by evaporating the solvent under reduced pressure to get the compounds **2a–c** in 70–80% yield as oil, which were used for *polymerization without further delay.*

2.4. *General procedure for copolymerization of amino diesters 2a-c with poly(ethylene glycol)*

Compound **2a–c** (0.01 mol) and PEG 600 (0.01 mol) were placed in a round bottom flask and temperature was raised to 60°C to make the reaction mixture homogeneous. Vacuum was applied to dry the reactants before use. Enzyme (10% by weight wrt reactants) was added to the above mixture and the reaction was allowed to proceed with continuous stirring under vacuum for 48 h, after which the reaction was quenched by adding 100 ml dichloromethane and removing the enzyme by filtration. The filtrate was dialyzed using a dialysis membrane (MWCO 2000) against 1000 ml of water for 24 h. After the completion of dialysis, the product was freeze dried to obtain brown viscous oil in 50–60% yield.

2.4.1. *Characterization of copolymer 3a*

^{1}H-NMR (δ_HCDCl$_3$, 200 MHz): 1.24 (t, 3H, COOCH$_2$C*H*$_3$ end group), 2.61(brs, 2H, N*H*$_2$, *D*$_2$*O exchangeable*), 3.41–3.70 (brs, 54H, OC*H*$_2$C*H*$_2$O PEG main chain), 3.71-4.28 (m, 7H, C*H*, 2×CHCOOC*H*$_2$ and COOC*H*$_2$CH$_3$ end group).

^{13}C-NMR (δ_cCDCl$_3$, 50 MHz): 14.44 (COOCH$_2$*C*H$_3$ end group), 58.76 (*C*HCOOCH$_2$), 62.0 (CH$_2$*C*H$_2$OH end group), 65.27 (CHCOO*C*H$_2$), 69.10 (CHCOOCH$_2$*C*H$_2$O), 70.58–71.69 (OC*H*$_2$C*H*$_2$O PEG main chain), 73.01 (*C*H$_2$CH$_2$OH end group), 169.79 (*C*OO), 166.08 (*C*OO end group).

IR (KBr) υ_{max}: 3490, 2872, 1741, 1687, 1648, 1456, 1350, 1294, 1251, 1108, 1038, 950, 852 cm^{-1}.

2.4.2. *Characterization of copolymer 3b*

^{1}H-NMR (δ_H CDCl$_3$, 200 MHz): 2.23 (brs, 2H, N*H*$_2$, *D*$_2$*O exchangeable*), 2.58–2.67 (m, 2H, CHC*H*$_2$COOCH$_2$), 3.45–3.71 (brs, 54H, OC*H*$_2$C*H*$_2$O of PEG main chain), 3.66–3.71 (m, 1H, C*H*NH$_2$), 4.08–4.15 (m, 4H, NH$_2$CHCOOC*H*$_2$ and CHCH$_2$COOC*H*$_2$).

^{13}C-NMR (δ_cCDCl$_3$, 50 MHz): 39.19 (CH*C*H$_2$COO), 51.5 (*C*HCH$_2$CO), 61.89 (CH$_2$*C*H$_2$OH), 64.19 (*O*CH$_2$), 69.27 (OCH$_2$), 70.5–70.87 (OC*H*$_2$C*H*$_2$O of PEG main chain), 73.06 (*C*H$_2$CH$_2$OH), 171.46 (*C*OO), 174.40 (*C*OO).

IR (KBr) υ_{max}: 3514, 2872, 1743, 1779, 1461, 1350, 1286, 1251, 1108, 1038, 950, 848 cm^{-1}.

2.4.3. *Characterization of copolymer 3c*

^{1}H-NMR (δ_HCDCl$_3$, 200 MHz): 1.24 (t, 3H, COOCH$_2$C*H*$_3$ end group), 2.19–2.51 (m, 4H, CHC*H*$_2$C*H*$_2$CO), 3.02 (brs, 2H, N*H*$_2$ *D*$_2$*O exchangeable*), 3.42-3.71 (brs, 55H, C*H*CH$_2$CH$_2$CO and OC*H*$_2$C*H*$_2$O of PEG main chain), 4.00–4.32 (m, 6H, CHCH$_2$CH$_2$COOC*H*$_2$, CHCOOC*H*$_2$ & COOC*H*$_2$CH$_3$ end group).

^{13}C-NMR (δ_cCDCl$_3$, 50 MHz): 14.42 (COOCH$_2$*C*H$_3$ end group), 25.22 (CH*C*H$_2$CH$_2$COO), 29.58 (CHCH$_2$ *C*H$_2$COO), 55.77 (*C*HCH$_2$CH$_2$COO), 62.01 (CH$_2$*C*H$_2$OH end group), 64.83 (O*C*H$_2$), 69.16 (O*C*H$_2$), 70.68-70.92 (OC*H*$_2$C*H*$_2$O of PEG main chain), 72.96 (*C*H$_2$CH$_2$OH), 166.12 (*C*OO end group), 172.58 (*C*OO), 178.26 (*C*OO).

IR (KBr) υ_{max}: 3476, 2872, 1744, 1702, 1456, 1350, 1286, 1250, 1105, 1038, 950, 848 cm^{-1}.

2.5. *Acylation of pendant amino group of copolymers 3a–c with nonanoyl chloride*

Polymer **3a–c** (0.001 mol) was dissolved in dichloromethane (40 ml) and placed in a round bottom flask under nitrogen. Triethylamine (0.125 g, 0.001 mol) was added, followed by the addition of nonanoyl chloride (0.20 g, 0.001 mol) dropwise at room temperature. The reaction mixture was left on stirring for 5–6 h. The solvent was evaporated and product obtained was washed with hexane three times to eliminate unreacted nonanoyl chloride. The crude product thus obtained was dissolved in tetrahydrofuran to precipitate triethylamine hydrochloride salt, which was filtered off and tetrahydrofuran was evaporated to obtain product as brown viscous oil in 85–95% yield.

2.6. *Characterization of polymer 4a*

^{1}H-NMR (δ_HCDCl$_3$, 200 MHz): 0.88 (t, 6H, *H*-9′ and *H*-9″), 1.28 (brs, 20H, *H*-4′-8′ and *H*-4″-8″], 1.63 (m, 4H, *H*-3′ and *H*-3″), 2.29 (t, 4H, *H*-2′ and *H*-2″), 3.45-3.72 (brs, 54H, OCH_2CH_2O of PEG main chain), 4.12–4.33 (m, 8H, 2×CHCOOCH_2, H-α and COOCH_2CH$_3$ end group), 5.24 (brs, 1H, *CH*NHCO), 6.71 (brs, 1H, CHN*H*CO, *D_2O exchangeable*).

^{13}C-NMR (δ_cCDCl$_3$, 50 MHz): 14.4(*C*H$_3$), 23.0 (*C*H$_2$), 25.74 (*C*H$_2$), 29.49 (*C*H$_2$), 29.58 (*C*H$_2$), 29.67 (*C*H$_2$), 32.18 (*C*H$_2$), 36.35 (*C*H$_2$), 56.59 (*C*HNHCO), 62.06 (CH$_2$*C*H$_2$OH end group), 65.82 (O*C*H$_2$), 68.97 (O*C*H$_2$), 70.70-70.93 (O*C*H$_2$*C*H$_2$O of PEG main chain), 72.94 (*C*H$_2$CH$_2$OH end group), 166.86 (*C*OO), 173.29 (NH*C*O).

IR (KBr) υ_{max}: 1736, 1686, 1456, 1350, 1296, 1251, 1105, 1040, 950, 854 cm^{-1}.

2.7. *Characterization of copolymer 4b*

^{1}H-NMR (δ_HCDCl$_3$, 200 MHz): 0.86 (t, 6H, *H*-9′ and *H*-9″), 1.24 [(brs, 20H, *H*-4′-8′ and *H*-4″-8″], 1.60 (m, 4H, *H*-3′ and *H*-3″), 2.20 (t, 2H, *H*-2′), 2.30 (t, 2H, *H*-2″), 2.71–3.22 (m, 2H, NHCHCH_2), 3.51–3.73 (brs, 54H, OCH_2CH_2O of PEG main chain), 4.19–4.33 (brs, 6H, CHCOOCH_2, CHCH$_2$COOCH_2 and $H-\alpha$ end group), 4.86 (m, CH$_2$C*H*NHCO), 6.70 (brs, 1H, N*H*CO, *D_2O exchangeable*).

^{13}C-NMR (δ_cCDCl$_3$, 50 MHz): 14.48(*C*H$_3$), 23.0 (*C*H$_2$), 25.88 (*C*H$_2$), 25.98 (*C*H$_2$), 29.51 (*C*H$_2$), 29.60 (*C*H$_2$), 29.68 (*C*H$_2$), 32.18 (*C*H$_2$), 36.75 (*C*H$_2$), 48.82 (*C*HNHCO), 62.06 (CH$_2$*C*H$_2$OH end group), 63.92 (O*C*H$_2$), 65.08 (O*C*H$_2$), 68.33 (O*C*H$_2$), 69.16 (O*C*H$_2$), 70.72–70.93 (O*C*H$_2$*C*H$_2$O of PEG main chain), 72.94 (*C*H$_2$CH$_2$OH end group), 171.27 (*C*OO), 171.67 (*C*OO), 173.36 (NH*C*O).

IR (KBr) υ_{max}: 1736, 1686, 1456, 1350, 1296, 1250, 1106, 1038, 950, 854 cm^{-1}.

2.8. *Characterization of copolymer 4c*

^{1}H-NMR (δ_HCDCl$_3$, 200 MHz): 0.86 (t, 6H, *H*-9′ and *H*-9″), 1.24 [(brs, 20H, *H*-4′-8′ and *H*4″-8″], 1.60 (m, 4H, *H*-3′ and *H*-3″), 2.20-2.49 [m, 8H, CHCH_2CH_2COO, *H*-2′ and H-2″], 3.51–3.73 (brs, 54H, OCH_2CH_2O of PEG main chain), 4.18–4.23 (m, 4H, CHCH$_2$CH$_2$COOCH_2, and *H*-α end group), 4.23–4.40 (m, 3H, CHCOOCH_2, and C*H*), 4.86 (m, 1H, CH$_2$C*H*NHCO), 6.70 (d, 1H, NH, *D_2O exchangable*).

^{13}C-NMR (δ_cCDCl$_3$, 50 MHz): 14.48(*C*H$_3$), 23.0 (*C*H$_2$), 25.11 (*C*H$_2$), 25.26 (*C*H$_2$), 29.48 (*C*H$_2$), 29.58 (*C*H$_2$), 29.62 (*C*H$_2$), 32.16 (*C*H$_2$), 34.56 (*C*H$_2$), 55.86 (*C*HNHCO), 61.94 (CH$_2$*C*H$_2$OH end group), 63.72 (O*C*H$_2$), 64.79 (O*C*H$_2$), 69.14 (O*C*H$_2$), 69.55 (O*C*H$_2$), 70.53–70.89 (O*C*H$_2$*C*H$_2$O of PEG main chain), 73.13 (*C*H$_2$CH$_2$OH end group), 172.54 (*C*OO), 174.23 (NH*C*O).178.37 (*C*OO).

IR (KBr) υ_{max}: 1738, 1696, 1456, 1350, 1296, 1251, 1105, 1040, 950, 854 cm^{-1}.

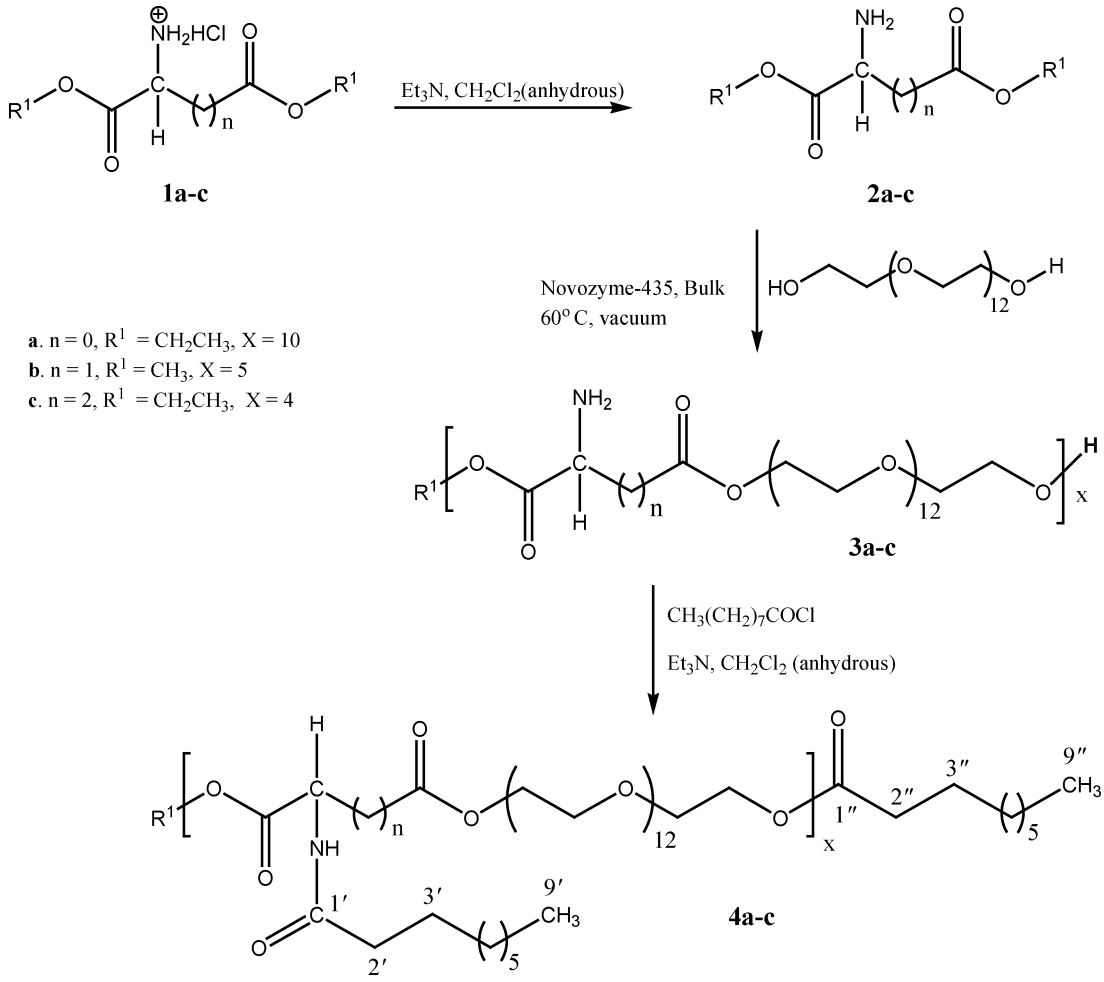
2.9. *Physical properties*

2.9.1. *Determination of aggregation, critical micelle concentration, particle size, particle distribution of polymer 4a–c*

The physical properties (R_g, CMC and M.Wt.) were measured by SLS and (R_h and particle distribution) by DLS. The CMC was measured by plotting the graph of scattering of light at 90°C vs. solution concentration. The aggregation behavior of polymers **4a–c** was determined by measuring the molecular weight of polymers in organic solvent as well as in deionized water. To perform these studies, the dust free solution of each of the polymers was made by dissolving the polymer in dichloromethane and filtered through 0.2 μm syringe filter. The solvent was then evaporated in dust free environment. The dust free sample was further used for making stock solutions in 0.02 μm pre-filtered toluene and deionized water. The stock solutions were used for evaluating the molecular weight by drawing the best fit Zimm plots, which produced 10–30 times higher molecular weight in deionized water as compared to toluene clearly indicating the aggregation behavior of polymers **4a–c** in aqueous solution. All these results have been summarized in Table 2.

3. Results and discussion

The structures of the copolymers formed by enzymatic condensation polymerization (Scheme 1) were analyzed by their ^{1}H and ^{13} C-NMR as shown in Figure 1 for copolymer **3a.** In ^{1}H-NMR spectra of **3a–c**, the signal at δ 4.15–4.40 was assigned to repeating methylene protons of ester linkage between ethoxy/methoxy carbonyl of amino diester and hydroxyl group of PEG. The formation of ester was further confirmed by lowering of signal intensity at δ



Sch. 1. Synthesis of copolymers 4a–c

1.30 ($-OCH_2CH_3$) due to the transesterification reaction in case of copolymers **3a** and **3c**. The remaining peak at δ1.30 ($-OCH_2CH_3$) was utilized as an end group to calculate the degree of polymerization. The broad signal at δ 3.60–3.80 was assigned to the main chain protons of the PEG. In the ^{13}C-NMR spectrum, the appearance of new signal at δ 64–66 ($-COOCH_2CH_2$) of copolymers **3a–c**, further confirmed the formation of polyester bond. To study the effect on polymerization and chemoselectivity, three amino diesters **2a–c** were selected. This selection was based on their sequential difference in diester positions, but the resultant polymers **3a–c** maintained the *regio/chemo* selectivity with all three monomers, **2a–c**. In order to prove the exclusive formation of polyester (*chemo*-selective) bond in the presence of free amino group, the ^{1}H and ^{13} C-NMR signal of ($>CHNH_2$), which is directly attached to the amino group has been compared in polymer and their respective monomer. The **DEPT** experiment was used to characterize the methine carbon of $>CHNH_2$ in the monomers, polymers and their acylated analogues. These results have also been compared in Table 1. It was confirmed that there was only one signal for the methine carbon of ($>CHNH_2$) in the carbon NMR spectrum which supported the selective transesterification leaving the amino group free. Whereas this methine proton and carbon was shifted from δ 3.3–4.1 to δ 4.6–5.2 in ^{1}H-NMR spectra and from δ 54.5–58.7 to δ 48.82–56.59 in ^{13}C-NMR spectra after acylation as shown in Table 1.

Although some protonation of free amino group in polymers **3a–c** was observed during dialysis, it was taken care of by using the excess of base during the acylation reaction to acylate all the amino groups. By taking advantage of this *regio/chemo* selective polymerization, the free amino group was utilized for attaching the hydrophobic chain to convert these copolymers into amphiphilic copolymers **4a–c** by using nonanoyl chloride (Scheme 1).

The properties of amphiphilic copolymers **4a–c** in aqueous solution showed that increasing the number of carbon spacer wrt α-amino ester in **2a–c**, resulted not only in an increase of the radius of gyration but also in critical micelle concentration. However, when the weight average molecular weight was measured in aqueous solution and compared

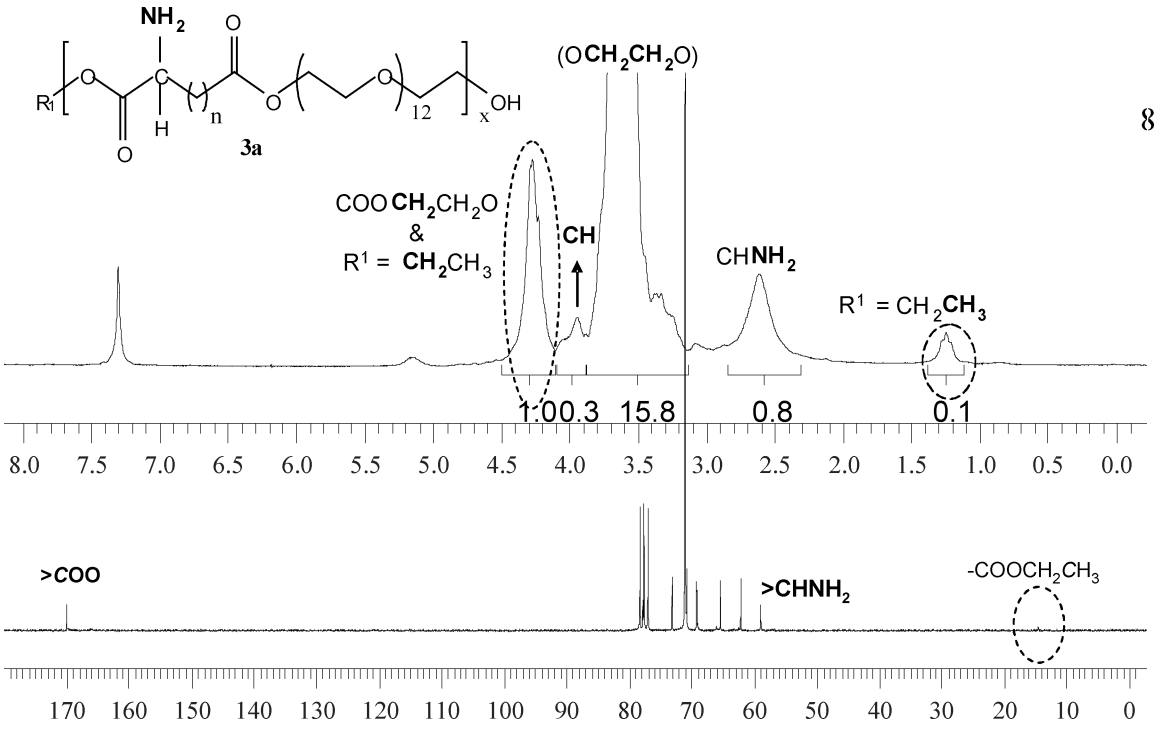


Fig. 1. Characterization of Polymer 3a by ^{1}H and ^{13}C-NMR.

Table 1. Chemical shift of CH of 2a–c, 3a–c and 4a–c. (*indicates overlapping signals)

Sample code	*Chemical shift in ^{13}C-NMR of CH (δ_c)*	*Chemical shift in ^{1}H-NMR of CH(δ_h)*
2a	58.7	3.9–4.1
3a	58.8	3.8–4.0
4a	56.6	5.1–5.2
2b	51.0	3.7–3.8
3b	51.5	*
4b	48.8	4.7–4.8
2c	54.5	3.2–3.3
3c	55.8	*
4c	55.9	*

Table 2. Molecular weight, critical micelle concentration and radius of gyration of polymers **4a–c**

Polymer code	*Mn in aqueous solution (Dalton) by SLS*	*Mn in Solvent (Dalton) by GPC/SLS*	*Rg (nm)*	*CMC (mg/ml)*
4a	$(4.820 \pm 0.112)10^4$	8,000	13.4 ± 3.4	0.832
4b	$(5.355 \pm 0.171)10^4$	3,500	18.8 ± 4.2	1.211
4c	$(9.372 \pm 0.544)10^4$	3,000	30.6 ± 3.5	1.68

with the molecular weight in organic solvent, it was found that they showed a reverse pattern i.e., unsymmetrical diesters showed high average molecular weight in aqueous solution whereas they were quite a bit less in organic solvent. This implies that unsymmetrical diester based polymers formed bigger aggregates.

4. Conclusions

In this study, various amino acid diesters were copolymerized with polyethylene glycol using lipase under solvent-less conditions. Lipase offered chemo/regio selective polymerization leaving a free amino group on back bone polymers. The synthesized copolymers were further converted to amphiphilic analogues using simple acylation of the free amino group. The physical properties of synthesized polymers were also measured to explore their ability for drug delivery and other biomedical applications.

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