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Synthesis, characterization, and *in vitro* drug release study of 3-arm poly-β-alanine

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ABSTRACT: Synthesis of three arms star-shaped poly-β-alanine (3-b-ala) based on tri(prop-2-yn-1-yl) benzene-1,3,5-tricarboxylate (TBT) and azido terminated poly-β-alanine (N_3-P-ala) was performed using click reaction. TBT was synthesized by nucleophilic substitution reaction between propargyl alcohol and 1,3,5-benzenetricarbonyltrichloride. For the first time, N_3-P-ala was synthesized through anionic polymerization of acrylamide using sodium azide as an initiator. TBT was characterized by FT-IR and ^{1}HNMR. N_3-p-ala was characterized by FT-IR, GPC, and ^{1}HNMR and 3-b-ala was characterized by FT-IR, GPC, ^{1}HNMR, TGA, and XRD. The synthesized 3-b-ala was used for drug loading and releasing studies. Polymer loaded drug (3-b-ala-D) hybrid was used in *in vitro* studies of drug (Diclofenac sodium) release in phosphate buffer solution (PBS) at 37 ± 0.5°C and pH 7.4. The drug loading and releasing studies were analyzed by UV-visible spectrophotometer. 3-b-ala-D was examined by AFM to analyze the surface morphology and roughness. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42124.

KEYWORDS: drug delivery systems; hydrophilic polymers; polyamides

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INTRODUCTION

Poly-β-alanine could be synthesized by the following processes: ring-opening polymerization of β-propiolactam and its derivatives in the presence of strong bases,[1–6] polycondensation of β-alanine in ionic liquid in the presence of triphenyl phosphite at higher temperatures,[7] elimination of HX form a β-alanine derivative[8] enzymatic catalyzed ring opening polymerization of substituted and unsubstituted β-lactam[9] and anionic polymerization of acrylamide in the presence of a strong base.[10–12]

In literature large numbers of studies on base-catalyzed anionic polymerization of acrylamide have been reported. This method was first patented in 1954 by Matlack and published in 1957 by Breslow *et al.*[10] According to the report, the initiation mechanism involved hydrogen abstraction by a basic initiator from the amide proton of acrylamide monomer and thus poly-β-alanine was obtained. Ogata[13] and Charles and Carraher[14] reported that the basic initiator binded with acrylamide monomer (Michael type addition) and an unstable intermediate was rearranged into stable amide anion, which further reacted with the acrylamide monomer.

In our present work, we first time reported the synthesis of poly-β-alanine through anionic polymerization of acrylamide by using sodium azide as an initiator. The anionic polymerization of acrylamide was performed by Michael type addition reaction and it was investigated by FT-IR and DFT calculation.

Diclofenac sodium is a potent non-steroidal anti-inflammatory drug, employed in the semi permanent treatment of inflammation and painful conditions of both rheumatic and non-rheumatic origin. Diclofenac sodium has limited water solubility, especially in gastric juices and is precarious in aqueous solution.[15]

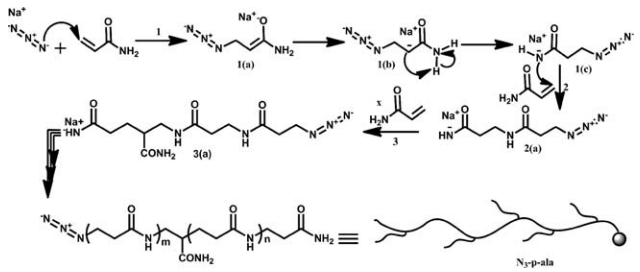
Azide terminated poly-β-alanine has immense benefit to modify its microstructure through azide-alkyne click reaction. Three arm poly-β-alanine and linear poly-β-alanine were employed as drug carriers for "*in vitro*" drug releasing studies. Three arm and linear poly-β-alanine were employed for sustainable release of hydrophobic drug (Diclofenac sodium). Polypeptide like structure present in poly-β-alanine is useful for biomedical application.

EXPERIMENTAL

Materials

Acrylamide (E. Merck, India), tetrahydrofuran (THF) (≥99%) (E. Merck, India), 1,3,5-benzenetricarbonyltrichloride (98% Sigma Aldrich, India), 1,10-phenanthroline monohydrate (Sigma Aldrich, India), propargyl alcohol (Avra, Hyderabad, India) and

Additional Supporting Information may be found in the online version of this article.



Scheme 1. Scheme and mechanism of anionic polymerization of acrylamide.

diclofenac sodium (Sigma Aldrich, India) were used as received. Copper (I) bromide (CuBr) (98% HIMEDIA, Mumbai) was purified before it is used. Acetone, dry *N*,*N*-dimethylformamide (DMF), potassium carbonate (anhydrous), sodium azide, and triethylamine (TEA) were supplied by S.D Fine Chemicals, Mumbai, India.

Anionic Polymerization of Acrylamide

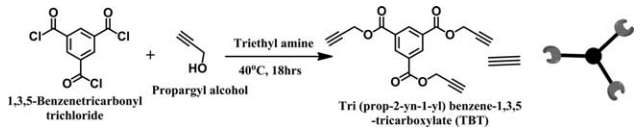
50 mmol (3.55 g) of monomer (acrylamide) and 1 mmol (65 mg) of initiator (sodium azide) were dissolved in 10 mL of DMF (dry) in a 100 mL of two-necked round bottom flask equipped with condenser and N_2 gas source. The reaction mixture was stirred at 70°C under N_2 atmosphere. After, 60 h the reaction mixture became viscous and the resulting polymer (N_3-p-ala) was precipitated and washed two times with acetone. Subsequently, polymer was dried in vacuum (10 mmHg) at 25°C for 48 h. Reaction and mechanism are shown in Scheme 1.Yield was observed to be 98.6%.

Synthesis of Tri(prop-2-yn-1-yl) benzene-1,3,5-tricarboxylate (TBT)

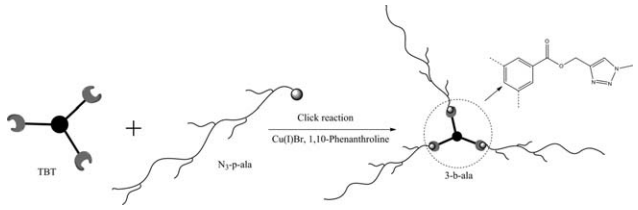
TBT was synthesized by the following method given in the literature.[16] 1,3,5-Benzenetricarbonyl trichloride was reacted with propargyl alcohol in the presence of triethylamine as a base. Further the formation of TBT was confirmed by the ^{1}HNMR and FT-IR studies. Reaction is shown in Scheme 2.

Click of (N_3–p-ala) with Tri(prop-2-yn-1-yl) benzene-1,3,5-tricarboxylate (TBT)

N_3-p-ala (4.516 g, 0.75 mmol [calculated by GPC analysis]), triprop-2-yn-1-yl benzene-1,3,5-tricarboxylate (0.81 g, 0.25 mmol) and 1,10-phenanthroline monohydrate (0.27 mg, 1.5 mmol) were dissolved in dry DMF (10 mL). CuBr (0.108 g, 0.75 mmol) was added under the protection of nitrogen gas flow. The reaction mixture was degassed by three freeze-pump-thaw cycles and then placed in an oil bath at 60 to 70°C with stirring for 24 h. The reaction mixture was exposed to air and the solvent was evaporated by vacuum rotary vapor. Then the mixture was washed several times with acetone. The 3-b-ala formed was packed in a dialysis membrane (cut off Mol. Wt. = 3000) and



Scheme 2. Synthesis of tri(prop-2-yn-1-yl) benzene-1,3,5-tricarboxylate (TBT).



Scheme 3. Click reaction of (N_3-p-ala) with (TBT).

packed polymer was immersed in water stream for 48 h to remove catalyst. The reaction mixture was reprecipitated in acetone and dried in vacuum (10 mmHg) at 25°C for 48 h and further polymer was used for characterization and drug release. Reaction is shown in Scheme 3. Yield was observed 61%.

CHARACTERIZATION

FT-IR

The spectrophotometer Perkin Elmer (spectrum 2) FT-IR was used to record IR spectra of N_3-p-ala, 3-b-ala, and TBT within the range of 4000 to 400 cm^{-1} by using solid state KBr pellet method. FT-IR spectrum of N_3-p-ala, 3-b-ala, and TBT are shown in Figure 1(a–c).

^{1}HNMR

The spectrometer FT-NMR JEOL AL 300 FT-NMR was used to record ^{1}HNMR of N_3-p-ala, 3-b-ala, and TBT in d_6-DMSO and $CDCl_3$, respectively. Tetramethylsilane was used as an internal reference. ^{1}HNMR spectrum of N_3-p-ala, 3-b-ala, and TBT are shown in Figure 2(a–c).

Thermal Analysis

TG analysis of 3-b-ala was recorded by TGA-DTA (Elmer-perkin STA 6000). TG analysis of the sample was carried out from 50 to 600°C, under inert atmosphere. The heating rate was 10°C per minute. The TG of 3-b-ala is shown in Figure 3.

GPC Analysis

The number-average molecular weight (M_n), weight-average molecular weight (M_w), and polydispersity index (M_w/M_n) of

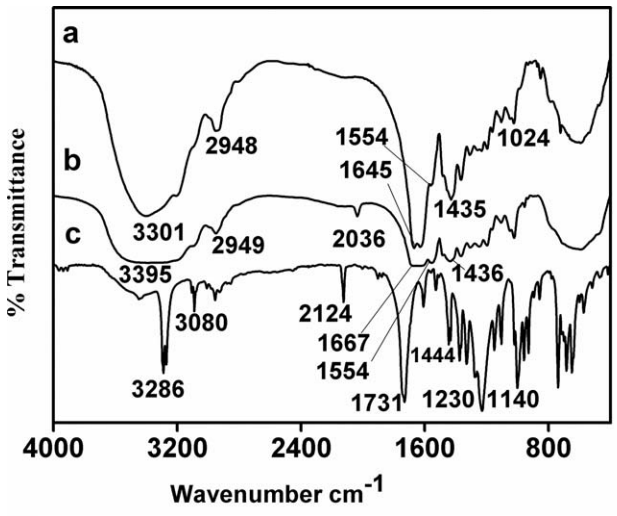


Figure 1. FT-IR spectrum of 1(a) 3-b-ala, 1(b) N_3-b-ala, and 1(c) TBT.

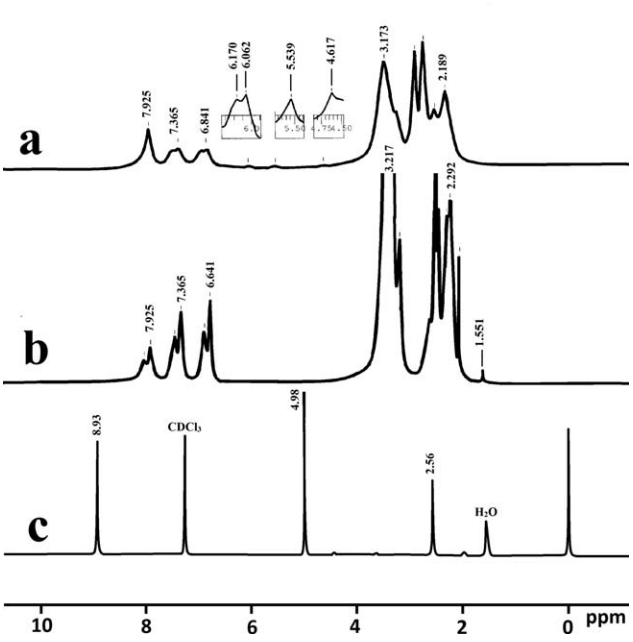


Figure 2. [1]HNMR spectrum of 2(a) 3-b-ala, 2(b) N_3-b-ala, and 2(c) TBT.

N_3-p-ala and 3-b-ala were determined by Youglin ACME 9000 Gel Permeation Chromatography in DMF at 40°C with flow rate 0.5 mL/min on two polystyrene gel columns [PL gel 5μm 10E 4 Å columns (300 × 7.5 mm)] connected in series to a Younglin ACME 9000 Gradient Pump and a Younglin ACME 9000 RI detector. The columns were calibrated against seven poly styrene (Pst) standard samples (Polymer Lab, Pst Calibration Kit, M-M-10). A detail of GPC is shown in supplementary information (Supporting Information Figure S1).

X-ray Diffraction

XRD analysis of 3-b-ala was performed by 18 KW Cu-rotating anode RIGAKU (Tokyo, Japan). The scattering angle (2θ) was varied from 5° to 70°. X-ray diffraction pattern is shown in Figure 4.

Preparation of Drug Carrier

Polymer and drug was taken in 10 : 1 proportion and mixed in water. Initially the solution was turbid but after few minutes it changed into a clear solution. Subsequently, the polymer-drug mixture was solidified by freeze dry and then co-precipitated by methanol. The resulting precipitate was used for "*in vitro*" drug release analysis in phosphate buffer solution.

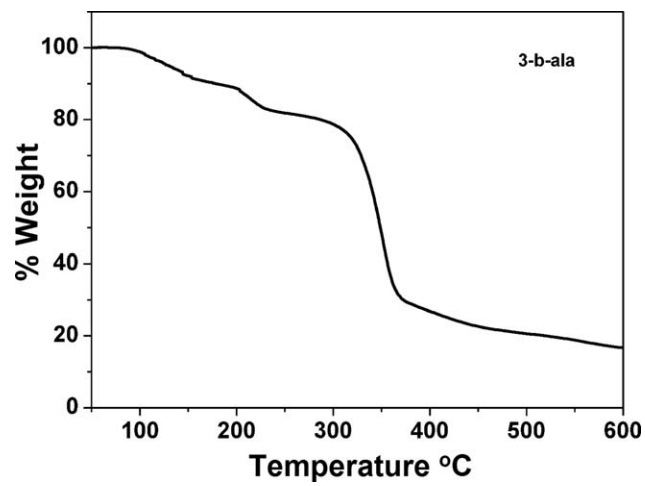


Figure 3. TG analysis of 3-b-ala.

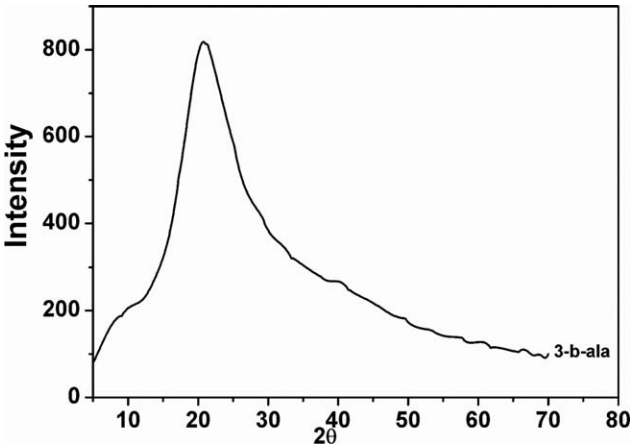


Figure 4. X-ray diffraction of 3-b-ala.

IN VITRO DRUG DISSOLUTION STUDIES

Preparation of Stock Solutions

From 1 mg/mL stock diclofenac sodium (DS) solution, 0.2, 0.1, 0.08, 0.06, 0.04, 0.02, and 0.01 mg/mL solutions were prepared by dilution and the characteristic absorbance peak of the drug was observed at 276 nm using a UV-Visible Spectrophotometer (Shimadzu UV-1700 pharmaspec, Kyoto, Japan). Finally, a calibration graph was plotted between concentration and absorbance. The chemical structure of diclofenac sodium is shown in Supporting Information Figure S2.

Procedure for Drug (Diclofenac Sodium) Release

Release of diclofenac sodium (DS) was determined by using a dialysis membrane of molecular weight cut-off 3000 Dalton. 3-b-ala loaded drug (3-b-ala-D) was packed inside and then immersed into 50 mL of 7.4 pH of phosphate buffer solution (PBS) and stirred at 50 rpm with temperature maintained in thermostat at 37 ± 0.5°C. Three milliliters of the sample was withdrawn at different time intervals, i.e., 0.167, 0.34, 0.5, 01, 02, 04, 06, 08, 10, 12, 14, 16, 18, 20, 22, 24, 25, 26, and 38 h and filtered through Whatman filter paper (GE Healthcare UK Ltd, UK) and the sample was exchanged by an equal volume (3 mL) of fresh PBS in every interval. Samples were analyzed for DS content using UV-Visible spectrograms. The percentage of DS release was reckoned from concentration versus absorbance calibration curve. The next interval exchanged solutions were analyzed by using same procedure as the drug content assay.[17]

Atomic Force Microscopy (AFM)

AFM images were recorded by NT-MDT Model Solver NEXT. Roughness was reckoned by a software NOVA Px 3.1.0 Rev 3880 which was provided by the manufacturer. The images were recorded in a semi contact mode by using a noncontact silicon cantilever (NSG10-DLC). 3-b-ala and 3-b-ala-D films were fabricated by dissolving 10 mg polymer sample in 100 μL water separately, to form clear solution. 20 μL of this solution was put on glass slide of approximate size (1 cm × 1 cm) allowing

for air dry followed by vacuum (10 mmHg) dry for 24 h before AFM observation. The roughness was determined with the root-mean-square (RMS) and average roughness of 10 × 10 μm scan areas of the respective materials.

RESULTS AND DISCUSSION

Anionic Polymerization of Acrylamide

Sodium azide has been used as an initiator for anionic polymerization of acrylamide for the first time. Azide ions have two terminal nitrogens with negative charge and one central nitrogen atom with positive charge. Over all the molecules, one anion is active species and other two neutralize each other. Azide ion initiates polymerization by Michael type addition reaction and the polymerization proceeds further as explained in the mechanism (Scheme 1). Thus, the resulting polymer was poly-β-alanine (N_3-p-ala) (GPC: $M_n = 4080$, $M_w = 6022$ and PDI = 1.47) with terminal azide group and it was confirmed by FT-IR, ^{1}HNMR, GPC, and DFT.

The mechanism of the reaction was also supported by density functional theory (DFT), where the geometries of intermediate 1(c) and initiation step were optimized at the RB3LYP/ 6–31G level in the gas phase using the Gaussian 03 package.43. Gaussview 3.09 was used to visualize the optimized molecular geometries. The calculations at this level provided evidence that the reaction proceeded by Michael type addition reaction between sodium azide and acrylamide in initiation step. In addition, close observation of the IR frequencies of the optimized molecule 1(c) and 2(a) predicted that an azide group was attached to the monomer unit and negative charge was generated on amide N atom. Details of computational studies are shown in Figure S3−S6 and Tables S1 and S2 in Supporting Information. Biodegradability test of N_3-P-ala is summarized in Supporting Information Table S3.

Click of (N_3-p-ala) with Tri(prop-2-yn-1-yl) benzene-1, 3, 5-tricarboxylate (TBT)

N_3-b-ala was used for post-polymerization modification to form three arm poly-β-alanine by click reaction with central moiety containing three alkyne groups (TBT) in the presence of Cu(I)Br/1,10-phenanthroline as catalyst. The resulting polymer (3-b-ala) was confirmed by FT-IR, ^{1}HNMR and GPC. The M_n, M_w and PDI of 3-b-ala were 6700, 8700, and 1.27, respectively.

FT-IR

FT-IR spectrum of N_3-b-ala is shown in Figure1(b). It has the characteristics stretching bands of N_3 stretching at 2036 cm^{-1}, —NH stretching at 3395 cm^{-1}, —CH stretching at 2949 cm^{-1}, >C═O stretching at 1667 cm^{-1}, —NH bending at 1554 cm^{-1}. Azide group stretching band proved that N_3-b-ala constituted N_3 group at terminal end.

FT-IR of TBT which is shown in Figure 1(c) has the characteristics bands of ≡CH stretching at 3286 cm^{-1}, aromatic CH stretching at 3008 cm^{-1}, —C≡C— stretching at 2124 cm^{-1}, C═O stretching at 1731 cm^{-1}, aromatic C═C stretching at 1444 cm^{-1}, C—O stretching at 1230 cm^{-1}. These characteristic bands support the formation of TBT.

In FT-IR spectrum of 3-b-ala, shown in Figure 1(a), combination of ≡CH, aromatic C—H and —NH stretching of amide group are observed at 3301 cm^{-1}. —CH stretching at 2948 cm^{-1}, >C═O stretching at 1645 cm^{-1}, —NH bending at 1554 cm^{-1} are also observed but no azide and alkyne stretching bands are present in the range of 2000 to 2100 cm^{-1}. Thus it is evident that post-polymerization modification has been done successfully through click reaction.

^{1}H-NMR

^{1}H-NMR of N_3-p-ala in d_6-DMSO is shown in Figure 2(b). In this spectrum the proton signals are observed as, N_3—CH (1.551 ppm, s, 2H), —NH—CH_2 (3.217 ppm, m, 2nH where n represents degree of polymerization i.e., 50), CH_2—C═O (2.292 ppm, m, 2nH), NH(t)—C═O (7.365 ppm, d, 1H), NH(c)—C═O (6.641 ppm, d, 1H) and NH—C═O (7.925 ppm, d, 1H). ^{1}HNMR proves that N_3-b-ala has been formed. N_3—CH proton peak is observed at 1.551 ppm which makes it evident that the polymerization has been initiated by azide ion. N_3-b-ala comprises of three variant amide-NH proton peaks and observed at 7.925, 7.365, and 6.641 ppm, due to secondary amide proton peak, primary amide (Trans) proton peak and primary amide (*cis*) proton peak respectively and their corresponding integral ratios were 1 : 1.62 : 1.82. Thus it is implied that synthesized N_3-b-ala constitutes linear and considerable branching units.

^{1}HNMR of TBT in $CDCl_3$ is shown in Figure 2(c). In this spectrum the characteristic alkynyl signal at 2.55 ppm can be seen clearly. The integration ratio of —C≡CH ($\delta = 2.56$ ppm), phenyl protons ($\delta = 8.93$ ppm) and —CH2—C≡C— ($\delta = 4.99$-4.98 ppm) has been calculated to be 1 : 1 : 2, indicating the formation of the desired product.

^{1}HNMR of 3-b-ala in d_6-DMSO is shown in Figure 2(a). Here the proton signals have been observed as, >N—CH (4.617 ppm, s, 2H), O═C—N—CH (3.173 ppm, m, 2H), CH—C═O (2.189 ppm, m, 2H), NH(t)-C═O (7.365 ppm, d, 1H), NH(c)-C═O (6.841 ppm, d, 1H) and NH-C═O (7.925 ppm, d, 1H) and O—CH (5.539 ppm, s, 2H). In 3-b-ala the N_3—CH proton peak of N_3-b-ala has been observed to be shifting from 1.551 ppm to 4.617 ppm, O—CH proton peak has been observed at 5.539 ppm and aromatic proton peak has been anticipated to come in the range of amide-NH proton peak area. All these evidences prove that postpolymerization modification has been successfully done by click reaction. Secondary amide proton peak, primary amide (trans) proton peak, and primary amide (*cis*) proton peak have been observed at 7.925, 7.365, and 6.841 ppm, respectively and their corresponding integral values ratios are 1.23 : 1.03 : 1.05, In case of N_3-b-ala the three variant amide —NH proton integral value is comparatively higher than 3-b-ala. It is evident that the branching units of polymer decreased and considerably linear N_3-b-ala has been clicked with TBT.

GPC Analysis of N_3-p-ala and 3-b-ala

N_3-p-ala (GPC: $M_n = 4080$, $M_w = 6022$, and PDI = 1.47) have branching units in polymer chains which has been analyzed by ^{1}HNMR. In 3-b-ala (GPC: $M_n = 6700$, $M_w = 8700$, and PDI = 1.27) the branching units reduced and this has been observed in ^{1}HNMR also. In GPC the M_n, M_w of 3-b-ala shifted to high molecular weight. From GPC data it can be inferred that the polymer contains mainly 3-arm poly-β-alanine and

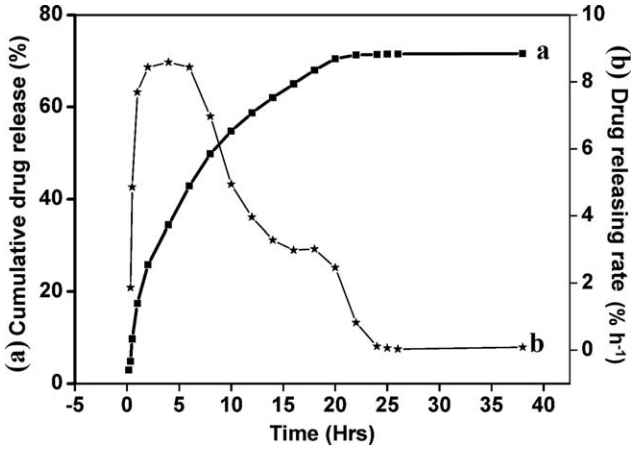


Figure 5. Cummulative percentage of diclofenac sodium released and releasing rate from 3-b-ala-D hybrid. 2.79% of drug loaded on 3-b-ala, drug loaded polymer hybrid was used in dissolution study at pH = 7.4 and 37 ± 0.5°C.

trace amounts of 2-arm poly-β-alanine and linear poly-β-alanine. Because of mixture of these three polymers, the molecular weight calculated through GPC was found less than three times.[18]

Thermal Analysis

First weight loss as observed in 3-b-ala at 190°C is due to split of polymer chains from central moiety. Second weight loss has been observed in 3-b-ala at 312°C due to dissociation of branching units from polymer chains. Degradation of polymer chains in 3-b-ala has been observed at 376°C.

X-ray Diffraction

XRD of 3-b-ala exhibited amorphous nature with its intensity peaks at 2θ value is 20.8.

Polymer-Drug Interaction

The polymer chains use their primary amide nitrogen (—$CONH_2$) to interact with inter molecular hydrogen bonding with secondary amide hydrogen of drug molecule. This has

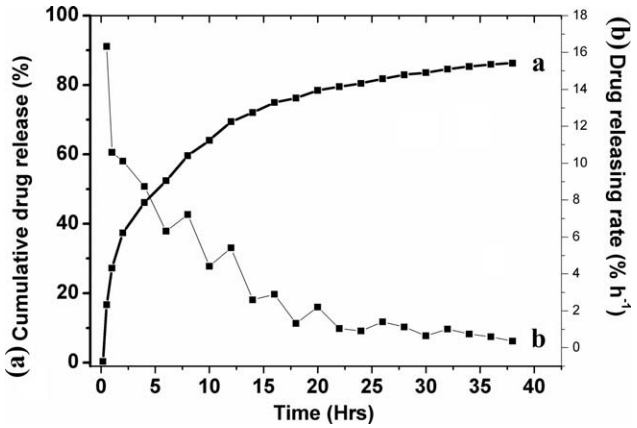


Figure 6. Cummulative percentage of Diclofenac Sodium released and releasing rate from N_3-P-ala-D hybrid. 3.396% of drug loaded on N_3-P-ala, drug loaded polymer hybrid was used in dissolution study at pH = 7.4 and 37±0.5°C.

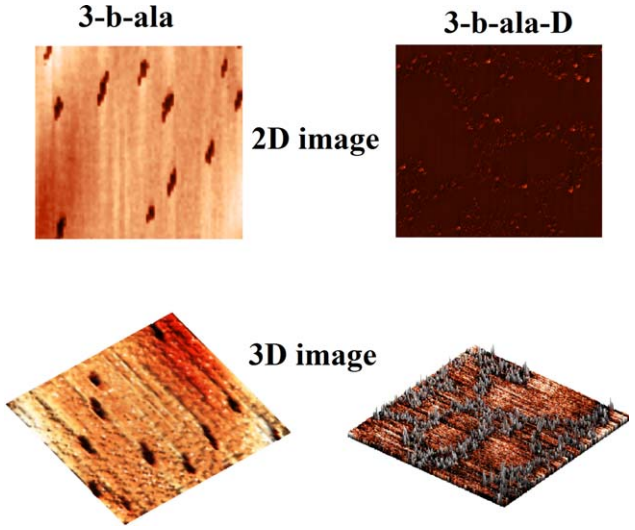


Figure 7. Two-dimensional and three-dimensional AFM images of 3-b-ala and 3-b-ala-D. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

been supported by IR and NMR analysis. Schematic representation of polymer-drug interaction is shown in Supporting Information Figure S7. IR analysis shows that amide I band is shifted to a lower region and in ^{1}HNMR investigation —CO—NH(c) proton peak is shifted to a higher chemical shift value. Thus the polymer primary amide nitrogen lone pair electrons are involved in hydrogen bond with secondary >N-H hydrogen atom of drugs. The IR and ^{1}HNMR of N_3-P-ala and N_3-P-ala-D are shown in Supporting Information Figures S8 and S9, respectively.

Drug Release Profile and Release Rate

The gained DS concentration in the medium of PBS, at every time interval, was studied by UV- visible spectroscopy through the characteristic absorption band of the drug at 276 nm. 13.95 mg DS was loaded on 500 mg of 3-b-ala, which was analyzed by calibration curve. The release profiles of the 2.79% w/w DS-loaded/3-b-ala hybrid in phosphate buffer solution are shown in Figure 5, where the drug releasing rate has been shown. In this experiment, drug release rate increases in first 6 h and afterwards decreases for each interval up to 16 h. Then there is slightly increase which subsequently decreases and becomes static after 24 h up to the observed time. In drug releasing profile, first 2 h of the releasing curve follows the first-order kinetics and 26% drug release. Afterwards it follows exponential growth up to 20 h and drug release 69.6%. Drug release later on the curve become static from 38 h and release is 71%. Standard deviation of drug releasing profile of 3-b-ala-D hybrid is shown in Supporting Information Figure S10 and statistical analysis of drug releasing profile I and II are summarized in Supporting Information Table S4.

Five hundred milligrams of N_3-P-ala was loaded with 16.98 mg of DS and it was analyzed by calibration curve; 3.396% w/w DS-loaded/N_3-P-ala hybrid drug release profile was analyzed in phosphate buffer solution at pH 7.4. Percentage drug release for N_3-P-ala was 86.26 in 38 h. The drug release profile and drug

releasing rate are shown in Figure 6. In this investigation, 37.37% drug was released in first 2 h and first-order kinetics was followed up to 4 h. Then it showed exponential growth up to 18 h and 76.26% drug was released. Afterwards % release became static up to the observed time. For first 4 h the drug release rate was higher due to the release of drug adsorbed on surface. The drug release rate of N_3-P-ala was observed to be more than 3-b-ala. Thus the branching in 3-b-ala held the drug tightly than linear poly-b-ala.

Atomic Force Microscopy (AFM)

The three-dimensional AFM images show the nature of height mode structures and surface morphology. AFM provides concurrent detection of height and surface roughness. The surface morphologies of 3-b-ala and 3-b-ala-D are shown in Figure 7. The surface root mean square and average roughness, of 3-b-ala is 1.768 nm and 0.813 nm and 3-b-ala-D is 11.494 nm and 6.945 nm, respectively. AFM calculated surface roughness shows that 3-b-ala-D has highest surface roughness and this implies that drug is carried by 3-b-ala more effectively. From the AFM analysis of 3-b-ala and 3-b-ala-D, visual evidence for the appearance of height mode structures has been observed.

CONCLUSIONS

N_3-p-ala was successfully synthesized by anionic polymerization of acrylamide with azide terminal and its greater advantage affirms to post polymerization modification. TBT was synthesized by nucleophilic substitution reaction. Synthesis of TBT was confirmed by FT-IR, ^{1}HNMR spectral studies. 3-b-ala was synthesized by click reaction between TBT and N_3-p-ala. FT-IR, ^{1}HNMR, and GPC characterizations proved that N_3-p-ala and 3-b-ala were successfully synthesized. Further 3-b-ala polymer was used as drug (DS) carrier and 2.79% (w/w) drug loading was observed on 3-b-ala, which had been proved by AFM study. 3-b-ala-D was studied for *in vitro* drug releasing in PBS and it was observed 71% drug released between 38 h.

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