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Degradable/cytocompatible and pH responsive amphiphilic conetwork gels based on agarose-graft copolymers and polycaprolactone

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Synthesis of polychloromethyl styrene (PCMSt)

First, CMSt was distilled under reduced pressure and then passed through neutral alumina coloumn to remove the inhibitor. A typical example of polymerization of CMSt is as follows. CMSt (10 mL), DMF (20 mL) and Azo bis isibutyronitrile (0.05 g) (AIBN) were taken in a round bottom flux. The reaction mixture was stirred at 70 ^{o}C for 3 h. The mass was precepitated in isopropanol. This procedure was repeated to remove unreacted monomer. The polymer was then dried in vaccum oven at 80 ^{O}C for 48 h. The monomer conversion was <60%. The ^{1}H NMR (Figure S1) indicated that the mole of monomer unit~mole of chloride in the polymer.

Synthesis of agarose-*g*-polymethyl methacrylate-*b*-poly(2-dimethylamino)ethyl methacrylate (Agr-*g*-PMMA-*b*-PDMA) by atom transfer radical polymerization (ATRP)

Two step polymerization: (i) using Agr-I as multifunctional macroinitiator for the ATRP of MMA to synthesize Agr-*g*-PMMA multifunctional macroinitiator and (ii) using Agr-*g*-PMMA as macroinitiator for the ATRP of DMA to synthesize targeted Agr-*g*-PMMA-*b*-PDMA . The synthesis of Agr-I and detailed characterization have been previously reported as discussed in the experimental section of main article.

A representative example of synthesis of Agr-*g*-PMMA (Agr:PMMA=78:22, w/w) is given below. Multifunctional macroinitiator Agr-I (3 g) was first dissolved in degassed NMP (50 mL) at 60 ^{o}C. Degaseed MMA (6 mL) was then added into the reaction vessel under nitrogen atmosphere. This solution was homogenized by stirring for 1 h. Afterwards, bpy (0.3 g, 0.002 mol) and CuBr (0.15 g, 0.001 mol) were added under nitrogen atmosphere. The reaction vessel was then closed with a rubber septum and was secured with Cu wire. The

mixture was stirred and ATRP was continued for 5 h at 45 ^{o}C to obtain limited conversion. The resultant viscous solution was diluted with NMP and was precipitated into excess methanol. The isolated mass was again re-dissolved in NMP and re-precipitated into excess methanol. The isolated polymer was washed again with methanol and dried for 40 h at 45 ^{o}C in vacuum. The % of grafting was calculated to be 28 by gravimetric process.

The synthesized Agr-g-PMMA (3 g) was dissolved in 30 mL degassed NMP. Next, DMA (5 g) previously purged with nitrogen was added into the reaction vessel under nitrogen atmosphere followed by the addition of bpy (0.1 g, 0.00068 mol) and CuBr (0.05 g, 0.00034 mol). The reaction vessel was then closed with a rubber septum which was secured with Cu wire. The reaction mixture was stirred at 35 ^{o}C for 3 h. The polymer was precipitated into petroleum ether, dried at 45 ^{o}C under vacuum. The graft copolymer was purified by dialysis technique. The copper free polymer was isolated from the water by freeze drying and the weight was taken after drying until constant weight was obtained. The graft copolymer thus isolated was characterized by GPC, IR, and NMR analyses. The graft copolymer is abbreviated as Agr_{115}-*g*-$PMMA_{47}$-*b*-$PDMA_{56.2}$-1 (Table 1, experimental section of main article).

Synthesis of Agr-g-PMMA-co-PDMA-2

Additionally, Agr grafted with random copolymer of PMMA and PDMA i.e. Agr-*g*-PMMA-*co*-PDMA-2 (Agr:PMMA-co-PDMA=33:67, w/w) was synthesized as follows. Multifunctional Agr-based macroinitiator (Agr-I, 3 g) was dissolve in degassed NMP (50 mL). Next, MMA (6 mL, 0.14 mol) and DMA (7 mL, mol) previously purged with nitrogen was added into the reaction vessel under nitrogen atmosphere and stirred for 2 h. Next bpy (0.3 g, 0.002 mol) and CuBr (0.15 g, 0.001 mol) were added under nitrogen atmosphere. The reaction vessel was then closed with a rubber septum which was secured with Cu wire. The reaction mixture was then stirred for 8 h at 45 ^{o}C to keep polymer yield limited. The copolymer was precipitated into excess methanol. The separated polymer was isolated, re-dissolved in NMP, and re-precipitated into excess methanol. The isolated polymer was washed again with methanol and dried for 40 h at 45 ^{o}C in vacuum. The monomer conversion

was 51% and %graft was calculated to be 212. The %graft was calculated by the following equation:

$$\% \text{ Graft} = \frac{\text{Weight of grafted chains}}{\text{Weight of backbone polymer (Agr)}} \times 100 \qquad (1)$$

The graft copolymer was dissolved in DMF and dialysed against water to remove rest of the copper complex. The purified graft copolymers were characterizaed by gel permeation chromatography (GPC), ^{1}H NMR, and IR. Molecular weight (M_n), polydispersity index (PDI), and degree of subtitutions of Agr were calculated by combination viscosity, GPC and by ^{1}H NMR.

Calculation of degree of initiator substitution in agarose-based initiator (Agr-I), M_n of PMMA and PDMA chains in Agr-*g*-PMMA-*b*-PDMA by ^{1}H NMR

The degree of initiator substitution in Agr-I was obtained by calculating molar ratio of initiator to Agr from ^{1}H NMR of Agr-I (Fig. S3) as follows:

degree of initiator substitution = $I_b/6I_a$, where I_b is an integral area of the (C−Br)−(C$\underline{H}_3$)$_2$ protons (6H at δ 2 ppm) and I_a is an integral area of the O−CH−O proton (one proton at δ =5.2 ppm from glucose units of Agr). Degree of initiator substitution was calculated to be 0.23.

M_n of PDMA [M_n(PDMA)] was calculated by comparing the intensity ratio of proton signal due to O−C$\underline{H}$−O protons of Agr and side chain protons due to –COO-CH$_2$C$\underline{H}_2$-N(CH$_3$)$_2$ units (Figure S3) of PDMA by the following equation:

$$M_n(\text{PDMA}) = \frac{R_{Agr} \times I_c \times 157}{2 \times I_a} \qquad (2)$$

where R_{Agr} is the number of repeat unit of Agr obtained from the molecular weight of Agr (125000 g/mol) as measured from viscometry and PDI of the Agr. I_a and I_c are the integral area of O−C$\underline{H}$−O protons of Agr and side chain protons due to –COO-CH$_2$C$\underline{H}_2$-N(CH$_3$) units

of PDMA chains respectively (Figure S3, top spectrum). The number 157 is the molecular weight of DMA unit.

The M_n of PMMA [M_n(PMMA)] was determined by the following equation:

$$M_n(PMMA) = \frac{R_{Agr} \times (I_d - I_c) \times 100}{2 \times I_a} \qquad (3)$$

where I_d (signals area e+e', Figure S3, top spectrum) is the intergal area of backbone protons of C$\underline{H}_2$-C(CH)- of DMA units of PDMA and C$\underline{H}_2$-C(CH)- protons of MMA units of PMMA (Figure S3, top spectrum). Thus, I_d-I_c is the integral area of protons of C$\underline{H}_2$-C(CH)- of MMA units of PMMA chains.

Measurement of degree of swelling and percent of equilibrium swelling of APCN films

The DMF extracted conetworks films (5 cm x 5 cm) were weighed and transferred in mili-Q water of different pH. The extent of swelling was determined periodically (1 h intervals) by removing the films from water by removing the water adsorbed to the surfaces by blotting with tissue paper, and weighing. Equilibrium water swelling was recorded when the weight of the swollen films remained unchanged at temperature 30 ^{o}C. The swelling data were taken after 1 h of contact with water. This is because these conetworks attained equilibrium swelling within 1 h. The percent swelling (%S_w) was obtained by the following equation:

$$\% S_w = \frac{m_{sw} - m_{dry}}{m_{dry}} \times 100 \qquad (4)$$

where m_{sw} and m_{dry} are the masses of the water-swollen and the dry films, respectively. The degree of swelling (DS) in water was also calculated by the ratio of the masses of swollen conetwork to the dry conetwork. Toluene swelling was measured using the similar procedure as described above.

Degree of ionization (DI) and effective p*K*

The DI of each sample was calculated by the ratio of the number of HCl equivalents added in water to the number of DMA equivalents present in the sample. The equivalent DMA in the sample were obtained by (i) subtracting the mol% of DMA units which were removed due to removal of uncross-linked copolymers by DMF extraction and (ii) by subtracting the equivalent of CMSt units used for the preparation of conetwork. This is because of the fact that small amount of (5%, w/w) PCMSt was sufficient for cross-linking which completely reacted with DMA units due to much higher concentration of DMA unit in the conetwork than that of CMSt used for cross-linking. The IR spectra of the conetwoks indicated absence of vibration bands due to -CH_2-Cl attached to phenyl ring (vide infra). The calculation of DI was based on two assumptions: (i) DMA moieties remained un-charged in the conetworks and the water itself cannot cause ionization of the DMA units in the conetworks and (ii) all added HCl partitions almost exclusively into the gel phase rather than the supernatant solution phase as reported earlier. The effective p*K* of DMA units in each conetwork was obtained from hydrogen ion titration curve as the pH (of the supernatant solution) at 50% ionization. The degree of swelling was obtained from the ratio of swelled mass to dry mass of films.

Stress-strain property

Stress-strain property (stress and elongation at break) of APCN films (2.5 cm long, 0.35 cm width 0.18 mm thick) was determined by the ISO 527 S2 method using Zwick Roell Z2.5 tester with 20 mm/min crosshead speed. The testXpert II-V3.5 software was used for data analysis. Measurements were carried out with 3-4 sample films after equilibrating with water,

whose averages are reported. The water content of the samples was maintained by placing a wet tissue around the samples during measurements. The stress-strain property was measured when the APCN films reached equilibrium swelling keeping in mind the application of these APCN films in water system.

DSC and AFM analyses

DSC analyses were performed in a Netzsch DSC 204 F1 Phoenix instrument. Proteus 6.1.0d Software was used for data analysis. The temperature scale was calibrated by Cyclohexane and Indium. Thoroughly dried samples (14 mg) were heated from 20 ^{o}C to 130 ^{o}C at a rate of 10 ^{o}C/ min. The samples were then quenched to 20 ^{o}C at a rate of 5 ^{o}C/min after maintaining it at +130 ^{o}C for 1 min, and then the second heating was performed at a heating rate of 5 ^{o}C/min. Glass transition temperature (T_g) was recorded as the infection point of the heat-capacity jump from the second heating curve.

AFM measurements were performed in semi-contact mode using a Ntegra Aura (made by Nt-Mdt, Moscow) instrument at room temperature in air, employing. The "Nova" software was used for image analysis. The mixture of copolymer and halide terminated PCL or PCMSt were mixed and the reaction mixture was heated for 10 min. Then the reaction mixture was diluted to 1% by the addition of DMF followed by filtration. The drop of solution was deposited on freshly cleaved mica and was then heated at 70 ^{o}C for 12 h. After drying, the deposited film was kept inside $CaCl_2$ filled desiccator to avoid moisture contact for AFM analysis.

Determination of critical micellar concentration (CMC) of degraded species

The degraded mass (0.03 g) obtained after 35 days of degradation time at pH 5 was collected by freeze drying. The appropriate amount of degraded mass directly dissolved in water. CMC

of the degraded mass was determined by fluorescence method using pyrene as hydrophobic probe at temperature 30 ^{o}C.

CMC of the degraded masses obtained from the conetwork was determined by fluorescence spectroscopy. In this method, pyrene was used as fluorescence probe where pyrene was entrapped within the core of the micelle, molded by the degraded product of the conetworks. Fluorescence spectra were taken at different concentrations of degraded mass. Detailed experiment was carried out as follows. First, 0.025 ml of 10^{-4} (M) pyrene solution in dichloromethane (DCM) was taken and DCM was evaporated. Then 2 ml Millipore water was added to the pyrene and admixed properly. Fluorescence spectrum of that solution was recorded. Then the stock solution (3 g L^{-1}) of degraded mass was added in different portion into the above solution and fluorescence spectra were recorded each time. Five separate peaks were observed each time and intensity ratios of 1st (I_1) to 3rd (I_3) peak were calculated. The I_1/I_3 value decreased with addition of increased amount of solution of degraded mass. This addition of solution of degraded mass was continued until I_1/I_3 value reached a constant value. Then I_1/I_3 values were plotted against the concentration of degraded mass which provided sigmoidal curve. The CMC value was obtained by plotting the derivatives of I_1/I_3 to concentration with concentrations.

Loading and release of prednisolone acetate

Prednisolone acetate (0.5 g) was dissolved in acetone (50 mL). The APCN sample (0.5 g) was dipped in acetone containing the drug and gently stirred for 24 h at 25 ^{o}C. The drug adsorbed conetwork was then washed thoroughly with acetone to remove surface bound drug if any. The Drug loaded conetwork again washed with water and dried in vacuum oven. The weight of dried conetwork was then recorded. The total 100 mg of prednisolone acetate was entrapped in the 0.5 g of APCN.

The release study of drug was performed in water by pouring the sample (0.2 g) in dialysis tube (molecular weight cut-off:1200) containing phosphate buffer solution (PBS, 20 mL) of pHs 5 and 7.4 separately. The dialysis tube containing drug loaded conetwork and PBS was dialysed against PBS (100 mL) of same pH and incubated at 37 ^{o}C. The pH of the solutions was monitored every day. The PBS outside the dialysis tube was changed after certain times (each reading) to maintain sink condition. The concentration of drug inside and outside the dialysis tube was monitored by UV-Visible spectroscopy. This process keeps the drug in solution. A standard calibration curve was used to calculate the concentration of released drug. This typical experimental setup separates the outside water (final release medium) from degraded polymeric species. The concentration of drug at any time at outside the dialysis tube is the released drug which is free from the barrier of conetwork and degraded species. Each experiment was carried out in triplicate.

Loading and release of 5-fluorouracil

First, 5-fluorouracil (0.2 g) was dissolved in millipore water (25 mL) with stirring for 3 h. Then 5 mL of the solution was taken out and UV-visible spectra were recorded at different dilution. A representative water swollen $APCN_{graft\text{-}3a}$ film (0.65 g) was dipped into the solution for 24 h with stirring (100 rpm) at 25 ^{o}C. After absorption of the drug within the conetwork film, rest of the solution was taken into volumetric flask (100 mL). The loosely bounded drug on the conetwork film surface was washed with millipore water. The whole solution was taken into the previous volumetric flask and volume was recorded. UV spectra of this solution were recorded at different dilution and calculated the amount of drug loaded within the conetwork film by comparing the UV-visible spectra of the solutions recorded before drug loading. It was found that 32 mg of drug was loaded within the conetwork film.

Drug release experiment was carried out at two different PBS of pHs 5 and 7.4 at 37^{o}C. For release of drug, the conetwork film (0.25 g) was put into the dialysis tube

(molecular weight cut off 1200) containing of PBS (50 mL). Then it was dialyzed against PBS (150 mL) of same pH and allowed to keep with stirring (200 rpm). The solution was changed after certain time. After particular time interval, UV spectra of the solutions of both inside and outside the dialysis were recorded. After recording each UV spectra, solution at outside was replaced by fresh PBS (150 mL) of corresponding pH. The same process was repeated up to 400 h. Each experiment was carried out in triplicate.

Cell viability

Cytocompatibility of APCNs and its degraded species was evaluated in HeLa 299 cell line using MTT cytotoxicity assay. Briefly, HeLa cells (1×10^4 cells/well) were seeded per well in a 96-well plate and incubated in a CO_2 incubator at 37 °C for 24 h. particles and films of APCNs (25 mg/well) were then incubated with cells for 24 h at 37 °C. Cells treated with polystyrene were used as a control. After removing the supernatant from each well and washing twice by PBS, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) and 100 µL of fresh media was then introduced. After incubation for another 4 h, the resultant formazan crystals were dissolved in isopropyl alcohol (100 µl) and the absorbance intensity was measured by a microplate reader (Bio-RAD 680, USA) at 570 nm. All experiments were performed in triplicate, and the relative cell viability was expressed as shown in the following equation:

$$\text{Cell viability} = \frac{\text{Sample}_{570}}{\text{Control}_{570}}$$

Hemolysis Assay

Blood was obtained from the blood voluntary healthy volunteer. A total of 1 mL of blood (anti-coagulated) was centrifuged at 1500 rpm for10 min to obtain RBCs which were washed thrice with PBS to remove any adsorbed proteins. Then, 50 µL of RBCs were diluted to 10 mL with PBS to prepare RBC stock solution. APCNs (5 mg) were incubated with RBC stock

solution (100 μL) at 37 °C for 1 h at 120 rpm. After 1 h, the mixture was centrifuged at 1500 rpm for 5 min and the released hemoglobin in the supernatant was analyzed using UV−visible spectrophotometer at 540 nm. Percent hemolysis was calculated with respect to hemolysis caused by negative control (PBS) and positive control (1% Triton X-100), as shown in the following equation:

$$\text{Haemolysis (\%)} = \frac{\text{Sample}_{540} - \text{Negative control}_{540}}{\text{Positive control}_{540} - \text{Negative control}_{540}}$$

Table S1. Hydrodynamic diameter of the micelles formed by the degradation of APCN gels and CMC of the formed species.

APCN	Hydrodynamic diameter (nm)			CMC ($g.L^{-1}$) (pH 7.4)
	pH 5	pH 7.4	pH 9	
$APCN_{graft\text{-}3a}$	135	110	94	0.12
$APCN_{graft\text{-}3b}$	142	115	102	0.14

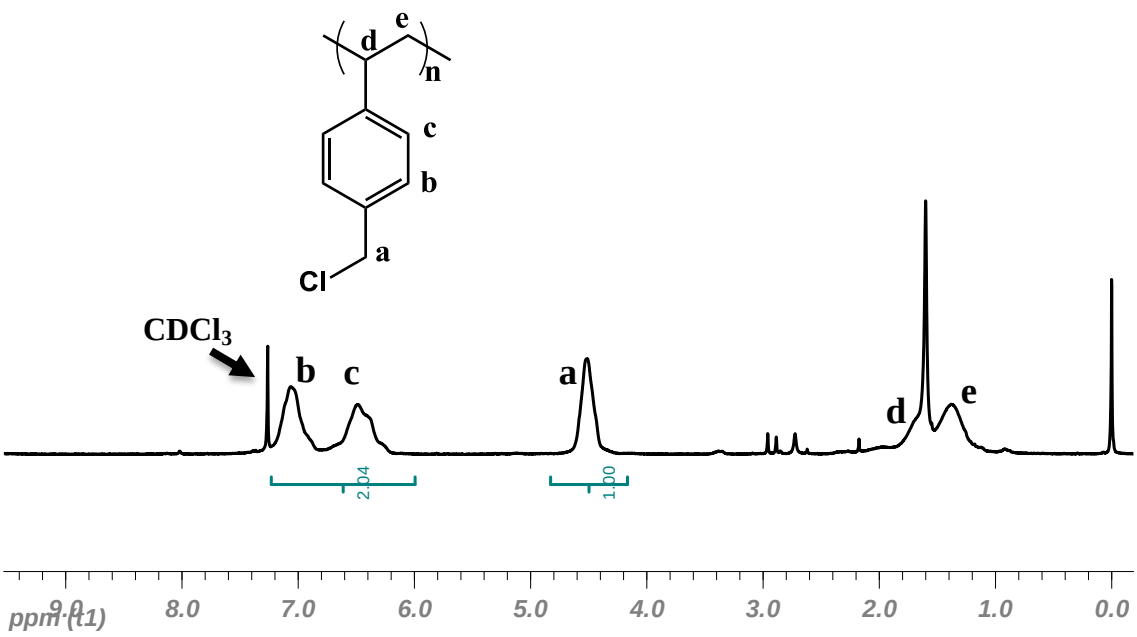

Fig. S1 ^{1}HNMR (200 MHz) spectrum of PCMSt. Spectrum was taken in $CDCl_3$.

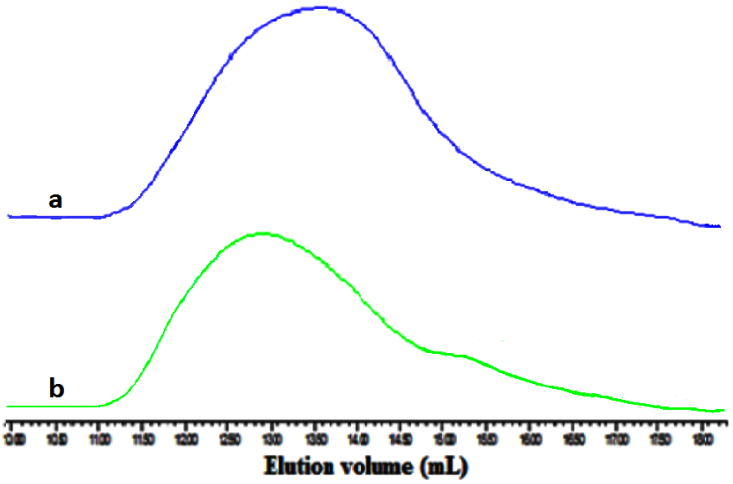

Fig. S2a GPC traces of (a) Agr-I and (b) Agr-*g*-PMMA-*b*-PDMA-3. GPC was carried out by using DMF as eluent at flow rate 0.8 mL/min.

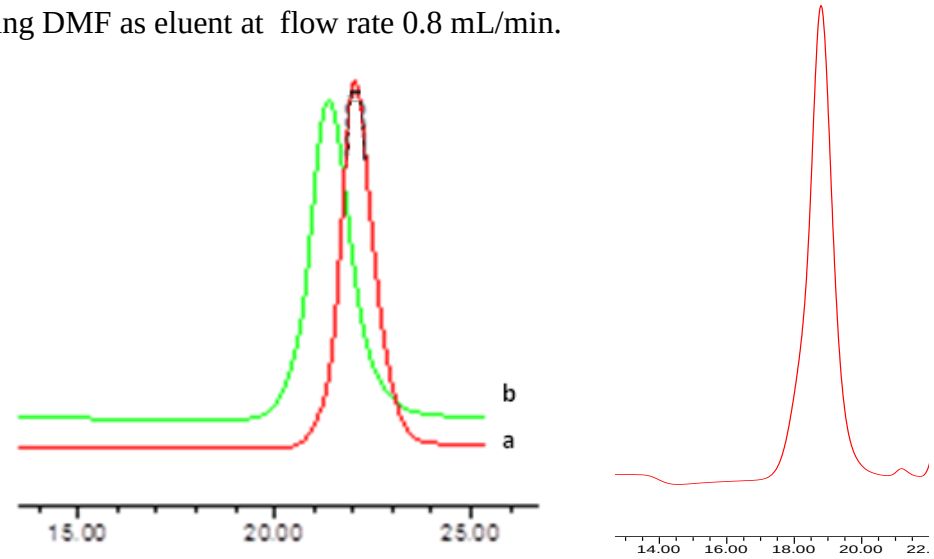

Fig. S2b Left: GPC traces of (a) PMMA macroinitiator and (b) PDMA-PMMA-PDMA-1. Right: GPC trace of Cl-CH_2-Ph-PCL-PhCH_2-Cl. GPC was carried out using THF as eluent at flow rate 1 mL/min.

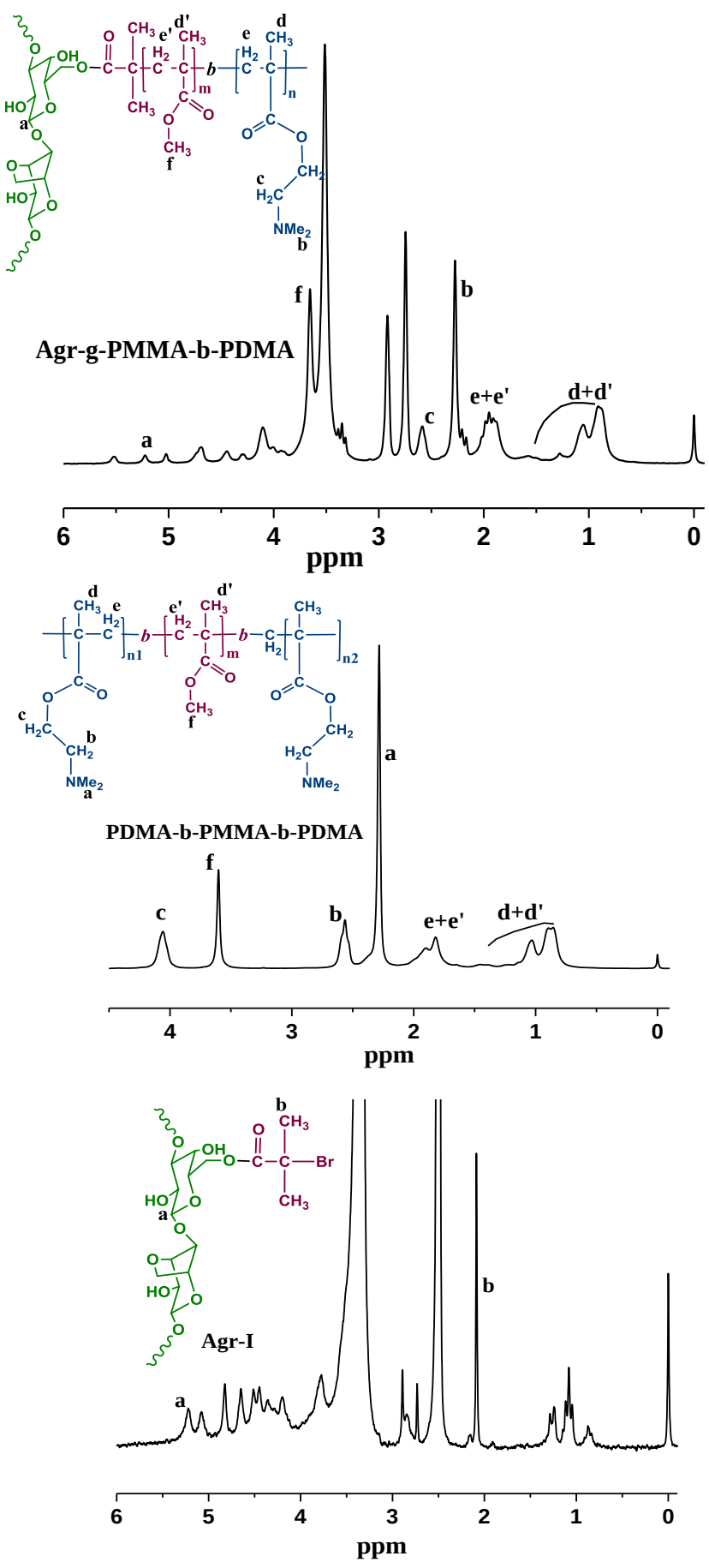

Fig. S3 Bottom to top are the ^{1}HNMR spectra (200 MHz) of Agr-I, representative PDMA-*b*-PMMA-*b*-PDMA and Agr-*g*-PMMA-*b*-PDMA-3. Spectra of Agr-I, Agr-g-PMMA-b-PDMA-3 and PDMA-b-PMMA-b-PDMA-1 were taken in DMSO-d6, DMF-d6 and $CDCl_3$ respectively.

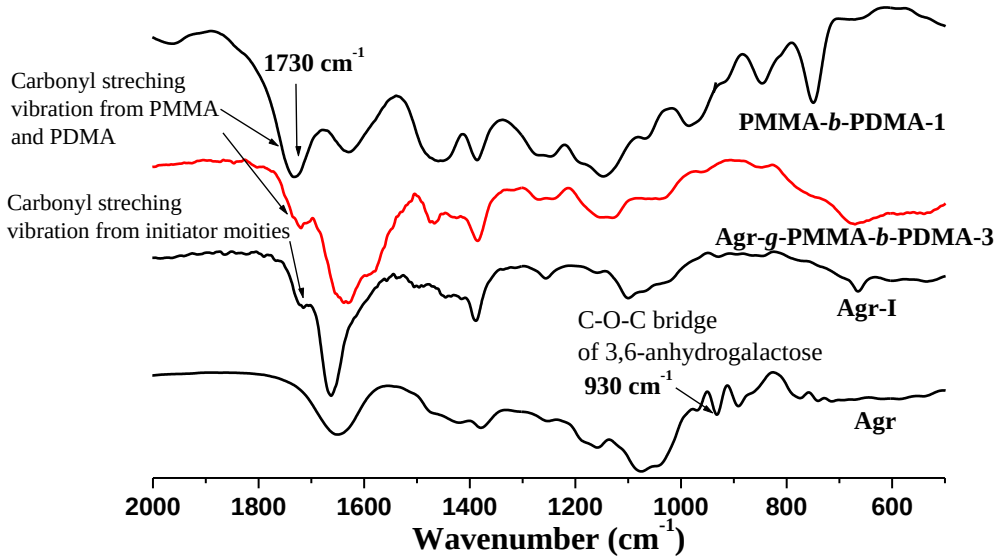

Fig. S4 IR spectra of Agr, Agr-I and two copolymers. The IR spectrum of Agr-I shows and additional band at ca. 1725 cm^{-1} due to –C=O stretching vibration owing to initiator moieties [-O-CO-C$(CH_3)_3$-Br] attached to Agr backbone. This band is absence in IR spectrum of neat Agr.

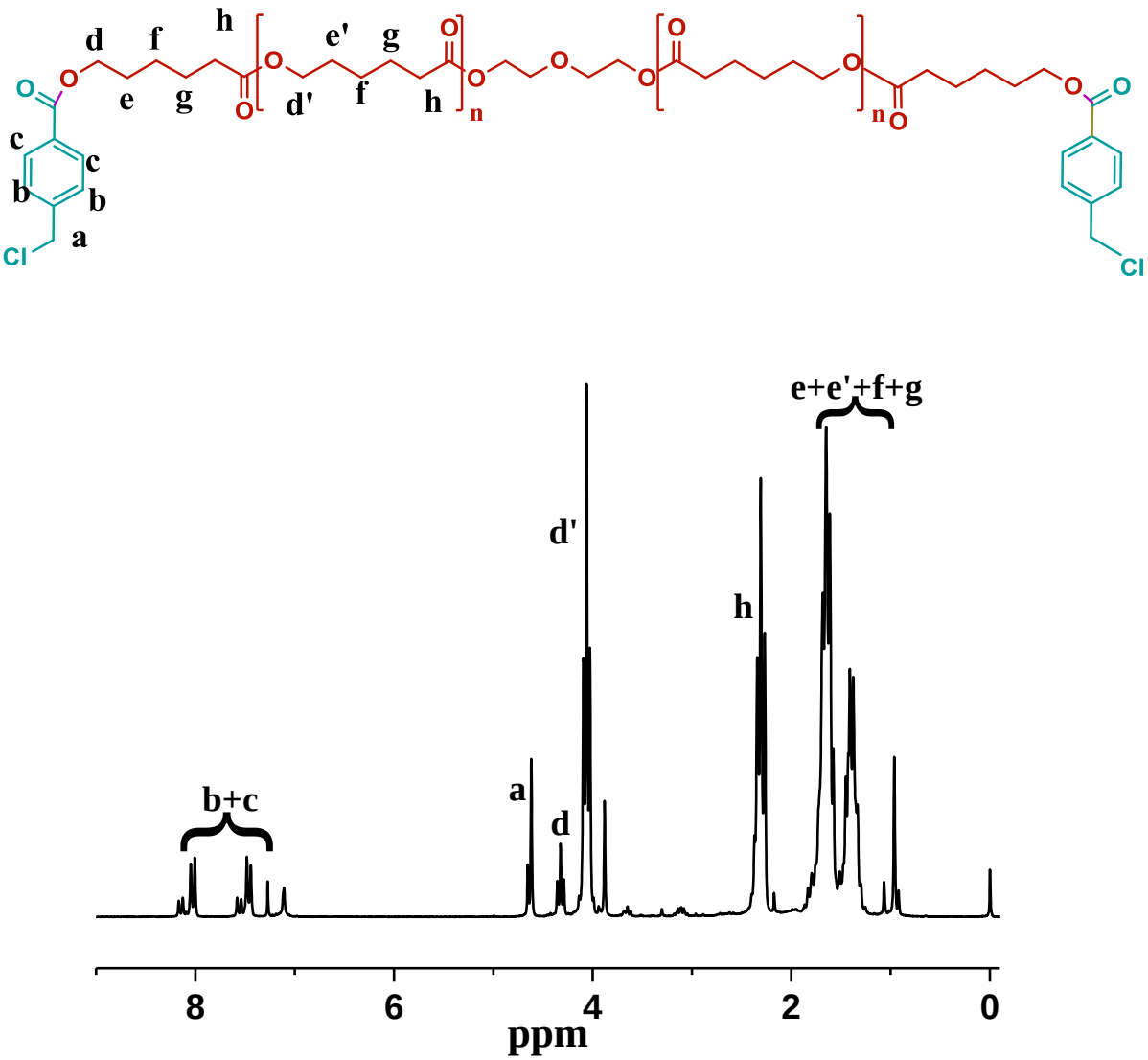

Fig. S5 ^{1}H NMR (200 MHz) spectrum of Cl-CH_2-Ph-PCL-PhCH_2-Cl recorded in $CDCl_3$.

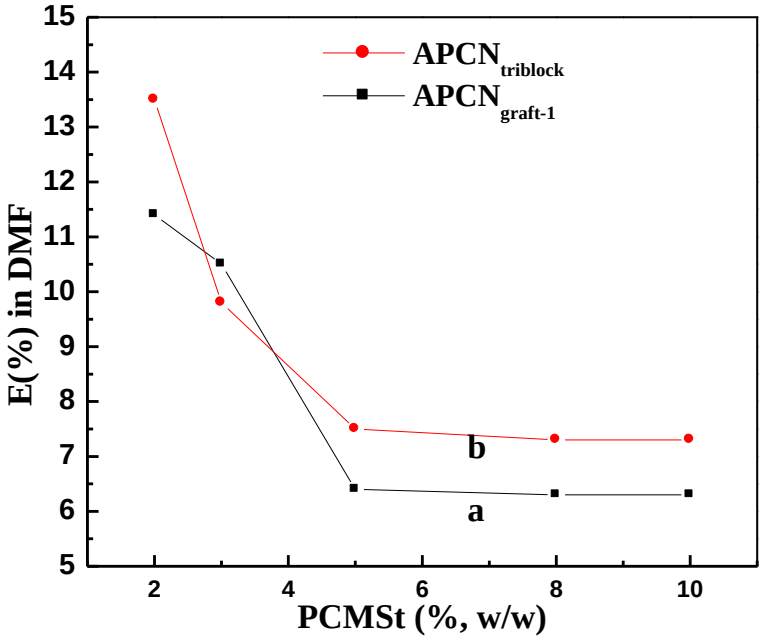

Fig. S6 Extractable obtained with conetworks prepared by reacting different amount of PCMSt with (a) Agr-g-PMMA-b-PDMA-1 and (b) PDMA-PMMA-b-PDMA-1.

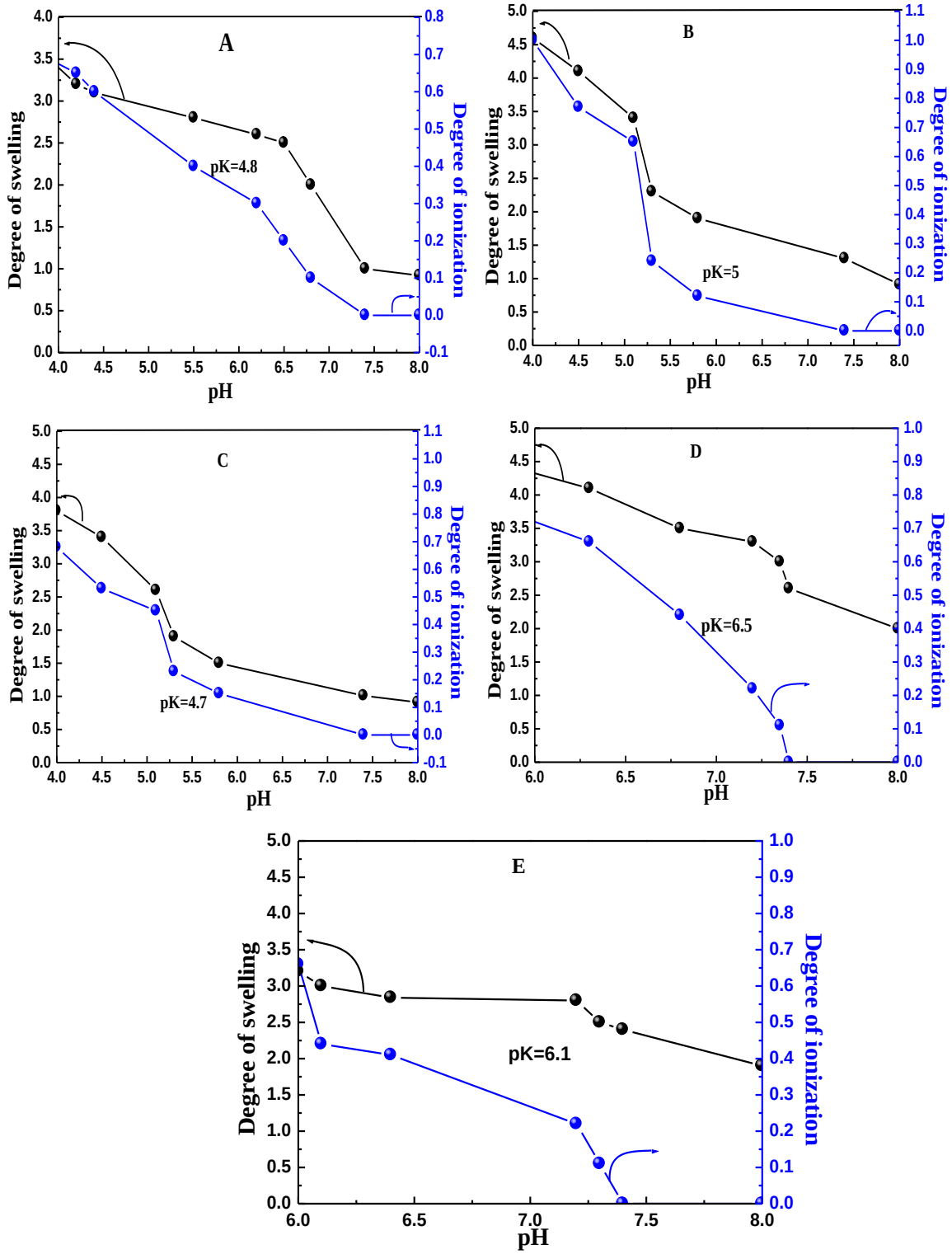

Fig. S7 Change of degree of equilibrium water swelling and degree of ionization with pH for (A) $APCN_{triblock\text{-}1a}$, (B) $APCN_{graft\text{-}1}$, (C) $APCN_{graft\text{-}2}$, (D) $APCN_{graft\text{-}3a}$ and (E) $APCN_{graft\text{-}3b}$.

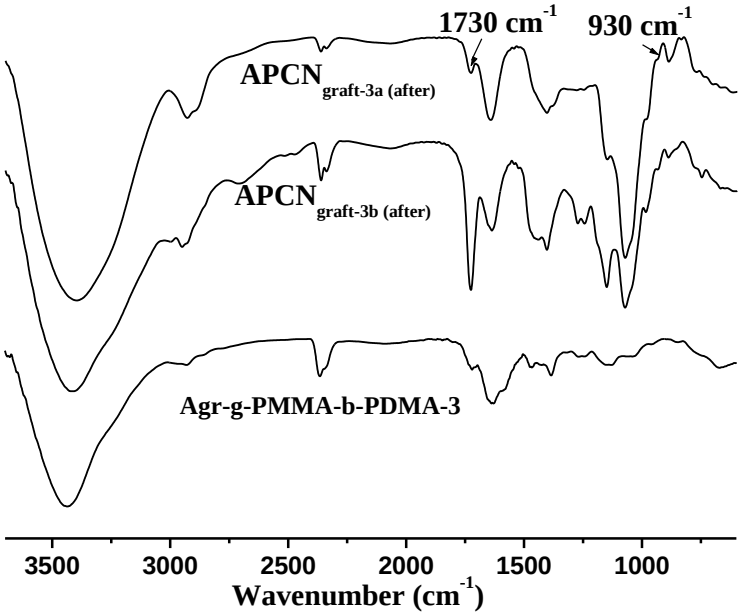

Fig. S8 IR spectra of Agr-g-PMMA-co-PDMA-3 copolymer and degraded masses of $APCN_{graft\text{-}3a}$ and $APCN_{graft\text{-}3b}$.

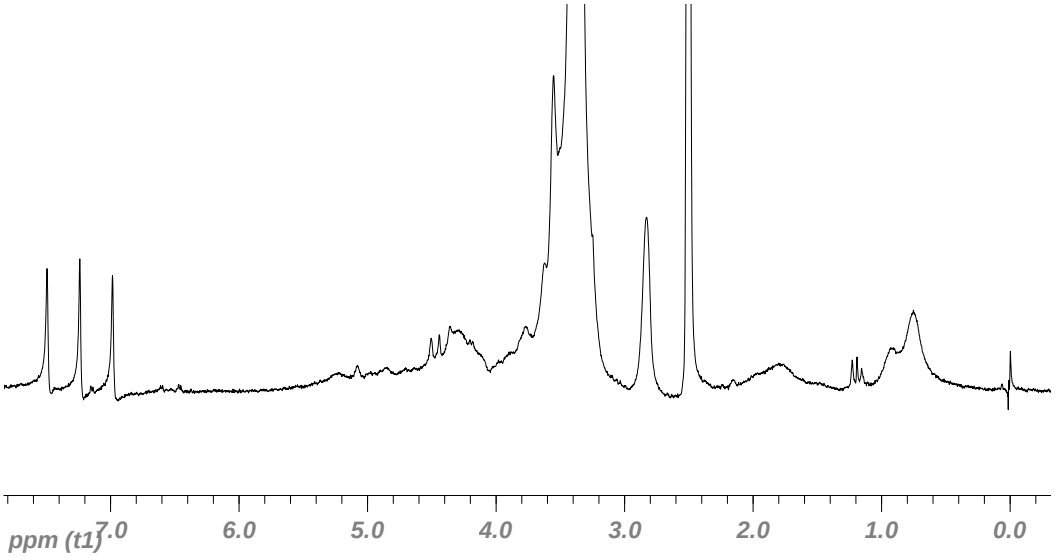

Fig. S9 ^{1}H NMR spectrum of degraded masses of $APCN_{graft\text{-}3a}$.

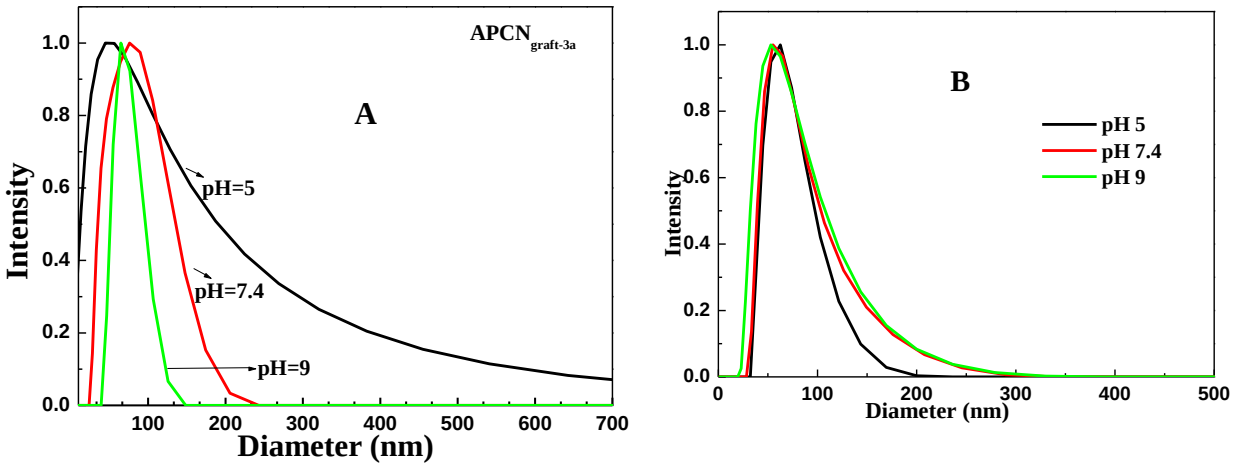

Fig. S10 DLS size distribution curves of micelles generated in the degraded medium from degradation of (A) $APCN_{graft\text{-}3a}$ and (B) $APCN_{graft\text{-}3b}$. Degradation was carried out at pH=5 for 35 days at temperature 37 ^{o}C. The pH of the solutions containing degraded species were then adjusted to 7.4 and 9.

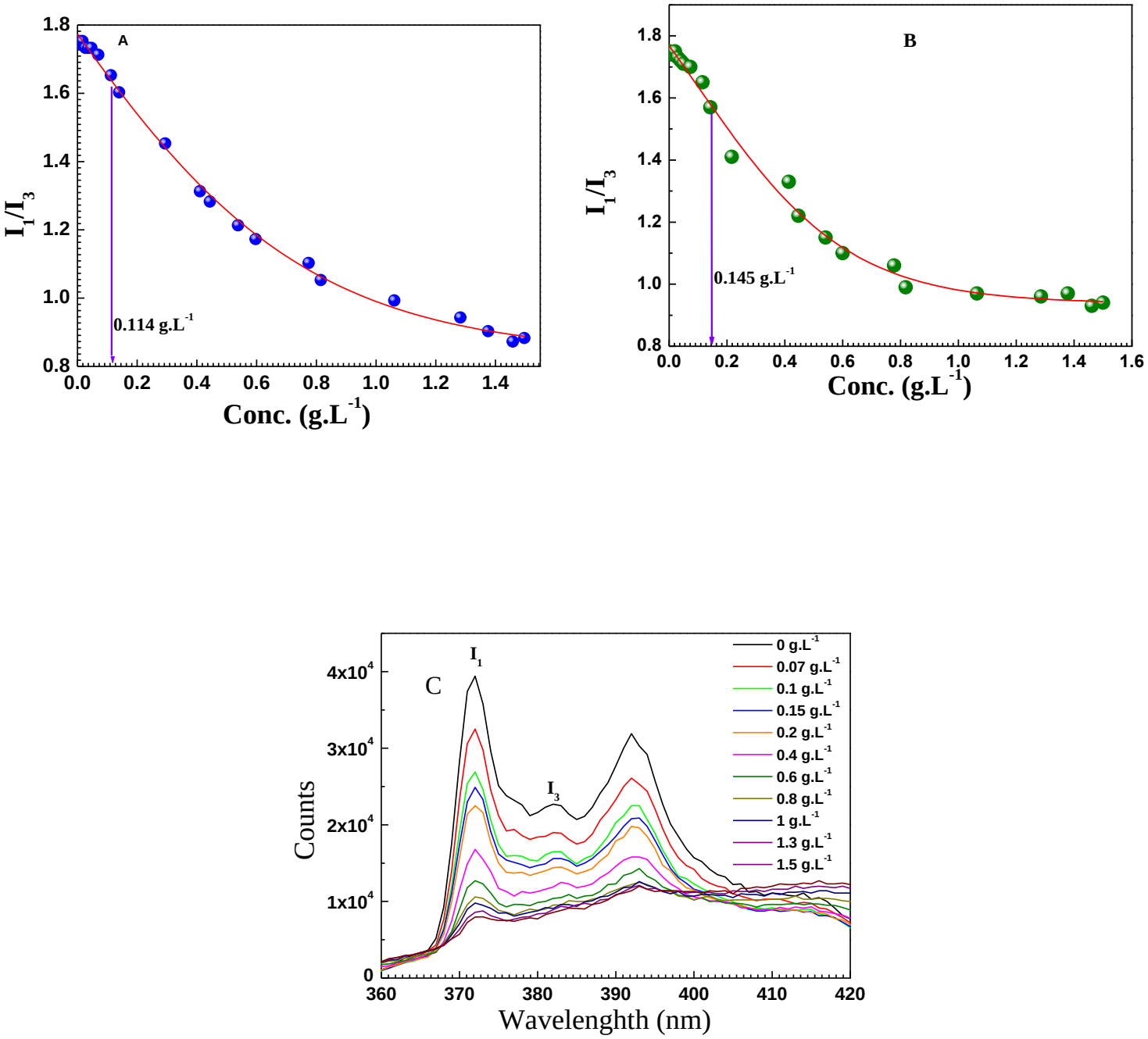

Fig. S11 Fluorescence intensity ratio (I_1/I_3) of pyrin vs concentration of degraded species of (A) $APCN_{graft\text{-}3a}$ and (B) $APCN_{graft\text{-}3b}$. Change of Fluorescence Spectra of pyrin (C) with concentration of degraded species of representative $APCN_{graft\text{-}3a}$. Degradation experiments were carried out at 37 ^{o}C, at pH 5 for 30 days. The fluorescence experiments were performed with the collected solid mass by dissolving in water.

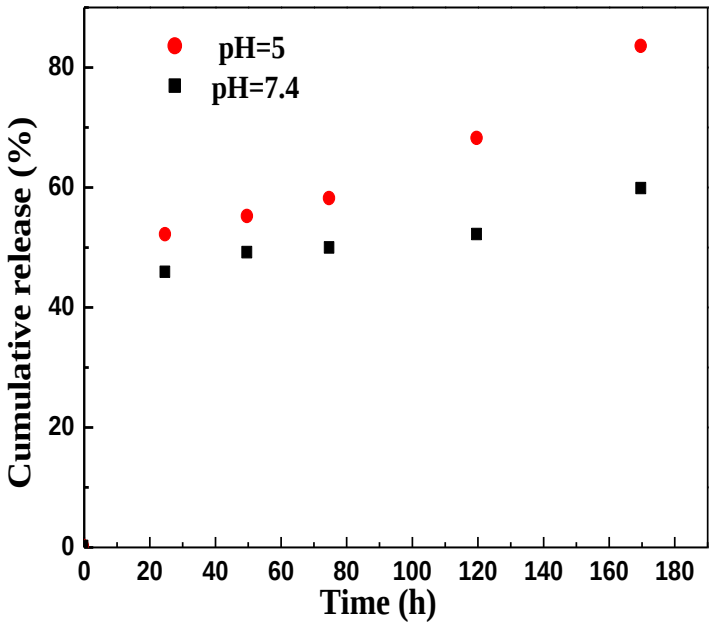

Fig. S12 Cumulative release of prednisolone acetate from micelles-entrapped drug generated after 350 h of release from degradation of $APCN_{graft\text{-}3a}$ during release study.