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ARTICLE

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

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Amphiphilic conetwork (APCN) gels have emerged as an important class of biomaterial due to their diverse applications. APCN gels based on biocompatible/biodegradable polymers are useful for controlled release and tissue engineering applications. Herein, we report a facile synthesis of APCN gel films by click type of sequential nucleophilic substitution reaction between pendent tertiary amine groups of agarose-*g*-poly(methyl methacrylate)-*b*(*co*)-poly(2-dimethylamino)ethyl methacrylate [Agr-*g*-PMMA-*b*(*co*)-PDMA] copolymers and activated benzyl chloride groups of polychloromethyl styrene or benzyl methyl chloride terminated polycaprolactone. A linear triblock copolymer (PDMA-*b*-PMMA-*b*-PDMA) containing central PMMA block and ends PDMA blocks was also employed for the synthesis of APCN gels for comparison purpose. These APCN gels exhibit co-continuous nanophase morphology, pH responsive water swelling and pH triggered release of hydrophobic and hydrophilic drugs. These gels are biodegradable/cytocompatible as confirmed by MTT assay and hemolysis experiment. The degraded species undergo micellization in aqueous environment and display low critical micelle concentration. Milled APCN gel particles are injectable through hypodermic syringe. This synthesis approach is extremely useful for preparation of library of APCN gels of diverse architectures and composition for biomedical applications.

Introduction

Amphiphilic conetwork (APCN) gel is composed of covalently linked hydrophilic and hydrophobic polymer chains.[1-7] APCN gels are mostly prepared by radical polymerization of telechelic macro-monomers containing at least two polymerizable groups with a selected low molecular weight monomer. APCN gels with a controlled structure were obtained by cross-linking well-defined polymer chains by end-linking using a di-functional monomers.[4-7] These polymeric conetworks have diverse applications such as nanotemplates for organic/inorganic nanohybrids,[8] pervaporation membranes,[9] membranes for water desalination via electrodyalysis,[10,11] supports for high efficiency enzyme catalysis,[12] antifouling/antimicrobial coatings,[13-15] biomaterials, such as controlled drug release matrices,[16,17] and scaffolds for tissue engineering.[18-20] Biostable/biocompatible APCNs had also been used for preparation of immunoisolatory device.[21,22]

A comparison may be made between APCN gel and hydrogel. APCN gel is a modified version of hydrogel which is useful for controlled release of both hydrophilic and hydrophobic drugs.[23] Hydrogel is mainly considered for the delivery of hydrophilic drugs.[24,25] This is because the release of drug generally governed by diffusion in the swollen polymer hydrogel having intrinsically high water affinity. This limits the hydrogel for the delivery of hydrophobic drugs. Mechanical property of water swollen polymer conetwork is also important for use in specific tissue engineering application. The advantages of APCN gel over simple hydrogel system are its superior mechanical property in water swollen state and controlled release behaviour of hydrophobic and hydrophilic drugs. This is due to relatively low degree of water swelling of APCN compared to hydrogel. It is noted that hydrogels have also been successfully used as tissue engineering scaffold[26] and regenerative medicine.[27]

As far as degradable APCNs is concerned, Rikkou et al. reported poly(methyl methacrylate) (PMMA) and poly(2-dimethylamino)ethyl methacrylate (PDMA) containing triblock and statistical copolymers-based APCNs.[28,29] These APCNs are degradable due to hydrolytic instability of the group transfer initiator or chain transfer agent (containing two cleavable hemiacetal ester groups) used for their synthesis. Polysaccharides are biocompatible and biodegradable polymers. Polysaccharides-based hydrogels are mostly reported which are formed by their cross-linking with different cross-linkers.[30-32] Injectable hydrogel made up of dextran and cyclodextrin was used for controlled delivery application.[33] Agarose (Agr) is an important naturally abundant polysaccharide extracted from cellular walls of agarophyte seaweed. Agr and its amphiphilic copolymers are useful for controlled drug delivery systems, microfluidics, cell culture substrates and cell encapsulation due to a high degree of biocompatibility.[34,35] Hence, it is highly desirable to synthesis Agr based APCN gel for biomedical applications.

Main objective of this work is the introduction of new approach for the synthesis of pH responsive and biocompatible/biodegradable APCN gels containing Agr as one of the components. Herein, we demonstrate the synthesis of APCN gels

in the form of flat films by click type of sequential nucleophilic substitution reactions between tertiary amine moieties of the Agr-based amphiphilic copolymers and polymer containing activated alkyl halide groups. For instance, we report a strategy for the synthesis of special APCN gels by sequential nucleophilic substitution reaction between activated benzyl chloride groups of poly(chloromethyl styrene) or benzyl methyl chloride terminated polycaprolactone and tertiary amine groups of Agr-*g*-PMMA-*b*-PDMA and Agr-*g*-PMMA-*co*-PDMA copolymers for pH triggered controlled release of hydrophobic and hydrophilic drugs. These APCN gels undergo degradation in aqueous environment and produced water soluble species which further undergo micellization in confined environment. The APCN gels and the species formed by degradation exhibited good cytocompatibility and hemocompatibility. The water swelled soft particles of milled APCN gels are injectable through hypodermic syringe.

Materials and Methods

Materials

MMA and DMA (98%, Aldrich) were distilled under reduced pressure and stored under nitrogen atmosphere at 5 ^{o}C. Acetone (GR, E-Merck, India), dimethylformamide (DMF) and N-methylpyrrolidone (NMP) (Fisher Scientific) were distilled. Bipyridyl (bpy, 98%), 2-bromoisobutyrobromide (iBuBr, 97%), Rose Bengal (RB) dye, hydroxyl-terminated polycaprolactone (HO-PCL-OH, M_n=2000 g/mol) and 4-(chloromethyl) benzoyl chloride (Cl-Bz-Cl, 98%) from Aldrich and were used as received. CuBr (98%, Aldrich) was purified by washing with 10% HBr in water followed by methanol and diethyl ether in a Schlenk tube under a nitrogen atmosphere. Chloromethyl styrene (CMSt, TCI, >90%) was distilled under reduced pressure and then pass through neutral alumina. Triethylamine (Et_3N), prednisolone acetate and 5-fluorouracil were from TCI and used as received. PCMSt was synthesized by free radical polymerization (ESI†) and characterized by ^{1}H NMR (Fig. S1, in ESI†). Agr (M_n~1.15x10^5 g/mol, PDI=1.9) grafted with block copolymer of PMMA and PDMA i.e. Agr-*g*-PMMA-*b*-PDMA copolymers with different compositions were synthesized by the use of Agr-based multifunctional ATRP initiator (Agr-I, degree of initiator substitution ~0.23) as described earlier by one of us (ESI†).[34] Additionally, Agr grafted with random copolymer of PMMA and PDMA i.e. Agr-*g*-PMMA-*co*-PDMA (Agr:PMMA-*co*-PDMA=33:67, w/w) was also synthesized (ESI†). All the purified graft copolymers were characterized by gel permeation chromatography (GPC, Fig. S2a, in ESI†), ^{1}H NMR (Fig. S3, in ESI†) and IR (Fig. S4, in ESI†). Molecular weight (M_n), polydispersity index (PDI), and degree of substitutions of Agr were calculated by combination of viscosity, GPC and by ^{1}H NMR analyses.[34] The molecular weights of PDMA and PMMA parts of the copolymers were obtained from ^{1}H NMR by comparing the integral ratio of proton signal due to Agr backbone and proton signals of PMMA and PDMA backbones (ESI†).

Preparation of benzyl methyl chloride-terminated polycaprolactone (Cl-CH$_2$-Ph-PCL-Ph-CH$_2$-Cl)

HO-PCL-OH (15 g, 0.0075 mol) was dissolved in dry toluene (80 mL) and about 20 mL of toluene was removed by rotary evaporator. The flask was capped by rubber septum under nitrogen atmosphere. The Cl-Bz-Cl (4 g, 0.02 mol) was dissolved in dry toluene (20 mL) and drop-wise added to the HO-PCL-OH solution. The reaction vessel was then kept in ice bath and Et_3N (2.9 mL, 0.02 mol) was added drop wise into the reaction mixture under vigorous stirring. After complete addition of Et_3N, reaction was allowed to continue for 24 h at 30 ^{o}C. The reaction mixture was then diluted with toluene and filtered off to remove the salt. Toluene was removed by rotary evaporator. Cold acetone was added into the collected mass and kept in freeze for 5 h. The precipitated white salt and unreacted excess Cl-Bz-Cl was filtered off. This procedure was repeated three times to remove the impurities. Acetone was then removed by rotary evaporator to collect Cl-CH$_2$-Ph-PCL-Ph-CH$_2$-Cl. The yield was ca. 95%. ^{1}H NMR, δ =1.42, 2.34, and 4.09 ppm are assigned to methylene protons of –(C<u>H</u>$_2$)$_3$–, –C<u>H</u>$_2$–CO–, and -C<u>H</u>$_2$OOC– in PCL units, respectively. The ^{1}H signals at δ = 4.25, 4.6 and 7-8 are for -C<u>H</u>$_2$OOC-Ph, -Ph-C<u>H</u>$_2$-Cl and aromatic protons of end groups of PCL (Fig. S5, in ESI†). The GPC trace (Fig. S2b, in ESI†) of Cl-CH$_2$-Ph-PCL-Ph-CH$_2$-Cl shows narrow PDI (PDI=1.2).

Synthesis of APCN gels

APCN gels were synthesized by reacting different copolymers with Cl-CH$_2$-Ph-PCL-Ph-CH$_2$-Cl or PCMSt. First, different amount of PCMSt was used to obtain minimum amount of PCMSt required for efficient cross-linking reaction. A representative example of synthesis of APCN with 5% (w/w) PCMSt is as follows. Purified Agr-*g*-PMMA-*b*-PDMA (1.9 g) was dissloved in DMF (25 mL) and strirred for 4 h. PCMSt (0.1 g) was separatley dissolved in DMF (1 mL). The PCMSt solution was then added into the the solution of copolymer under stirring at 30 ^{o}C. The complete transfer of PCMSt was ensured by transfering the left over solution by additional DMF (1 mL) immediately. The admixture was stirred for 10 min at 30 ^{o}C and then poured into flat petridish. The DMF was then evaporated at 60 ^{o}C for 8 h followed by drying in vaccum oven at 60 ^{o}C for 48 h to obtain dry APCN film. The thickness of the film was 0.2-0.25 mm. All the APCN films were prepared using copolymer to PCMSt ratio 95:5 (w/w) due to low extractables of the formed APCN gels with this precursors composition. A triblock copolymer (no Agr) (PDMA-*b*-PMMA-*b*-PDMA) was also allowed to react with PCMSt for synthesis of APCN gels.

APCN gels were also prepared by reacting graft and block copolymers with Cl-CH$_2$-Ph-PCL-Ph-CH$_2$-Cl using the same procedure except that equivalent amount (mol of CMSt unit) of Cl-CH$_2$-Ph-PCL-Ph-CH$_2$-Cl to that of PCMSt was used for cross-linking reaction. Table 1 summurizes the characteristics data of copolymers and abbreviations of corresponding APCN gels.

Table 1. Composition, M_n and PDI of precursors graft and triblock copolymers and abbreviations of corresponding APCN gels.

Copolymer	Agr/PMMA /PDMA^z (wt %)	PDI	APCN gel
PDMA$_{14.5}$-*b*-PMMA$_{11}$-*b*-PDMA$_{14.5}$-1	0/31/69	1.3	^{x}APCN$_{\text{triblock-1a}}$
PDMA$_{14.5}$-*b*-PMMA$_{11}$-*b*-PDMA$_{14.5}$-1	0/31/69	1.81	^{y}APCN$_{\text{triblock-1b}}$
Agr$_{115}$-*g*-PMMA$_{47}$-*b*-PDMA$_{56.2}$-1	60/20/20	1.73	^{x}APCN$_{\text{graft-1}}$
Agr$_{115}$-*g*-PMMA$_{40.4}$-*co*-PDMA$_{58.8}$-2	32/68	1.62	^{x}APCN$_{\text{graft-2}}$
Agr$_{115}$-*g*-PMMA$_{31.2}$-*b*-PDMA$_{54.5}$-3	59/15/26	1.68	^{x}APCN$_{\text{graft-3a}}$
Agr$_{115}$-*g*-PMMA$_{31.2}$-*b*-PDMA$_{54.5}$-3	59/15/26	1.7	^{y}APCN$_{\text{graft-3b}}$

x-PCMSt and y-ClCH$_2$Ph-PCL-PhCH$_2$Cl; APCN gels synthesized by reacting copolymers and PCMSt (copolymer:PCMSt=95:5, w/w) or ClCH$_2$Ph-PCL-PhCH$_2$Cl (copolymer:ClCH$_2$Ph-PCL-PhCH$_2$Cl=68:32, w/w); z-gravimetric analysis; subscript indicates M_nx10^{-3} of total grafting chains of PMMA or PDMA determined from ^{1}H NMR while the M_n of Agr was obtained from GPC and viscosity measurements.

Characterizations

Determination of extractable (E), measurements of degree of swelling, percent of equilibrium swelling, degree of ionization, effective p*K*, stress-strain property, differential scanning calorimetry (DSC) and atomic force microscopy (AFM)

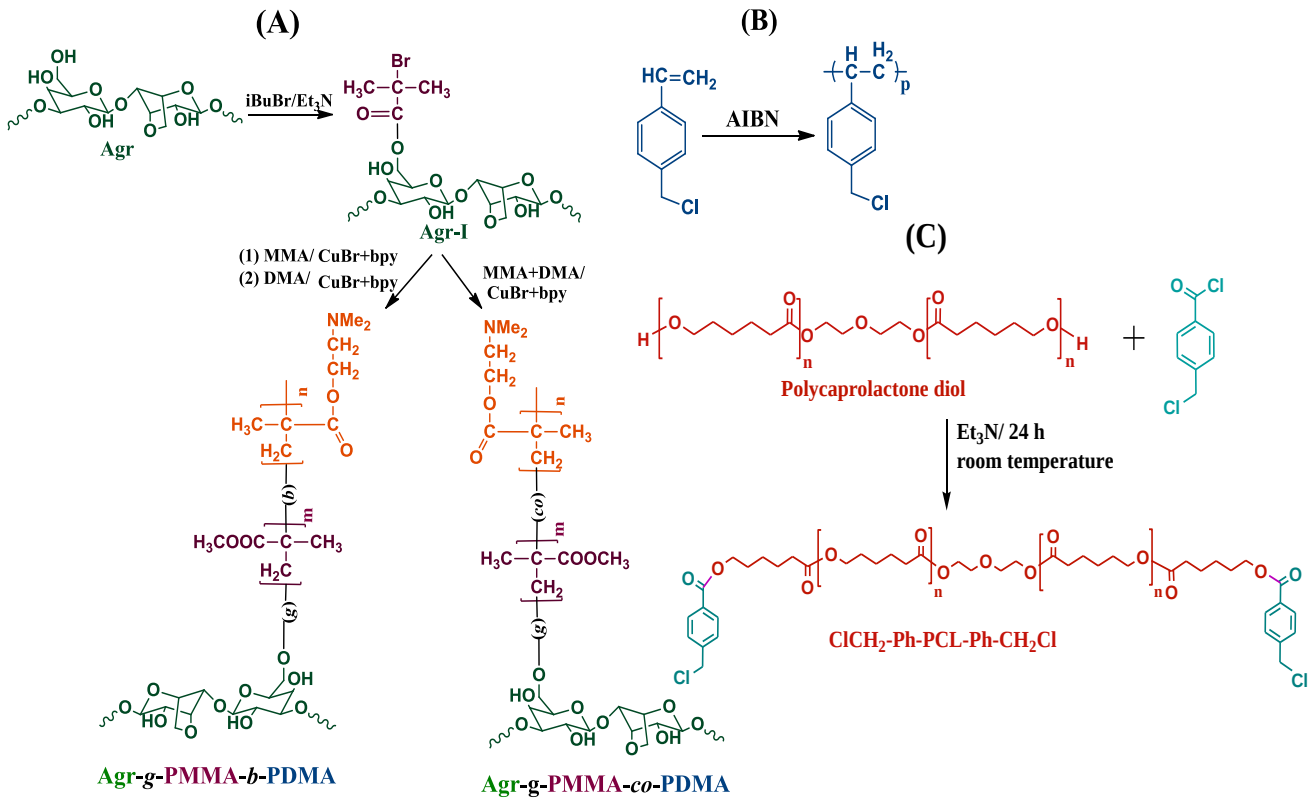
The dried APCN gel films (5 cm x 5 cm) in triplicate were weighed and then submerged in DMF (100 mL) and the container was shaken gently for 48 h at 30 ^{o}C. The films were then gently removed and washed thrice with DMF and water. The films were dried at room temperature and extracted with methanol to remove entrapped water. The films were then dried under vacuum at 70 ^{o}C overnight and weighed. The percent of extractable (%E) was calculated by the following equation:[22]

$$\%\,E = \frac{m_d - m_{ex}}{m_d} X\ 100 \quad (1)$$

where m_d and m_{ex} are the masses of the virgin and extracted samples, respectively. Averages of three determinations are reported. Degree of swelling, equilibrium swelling, degree of ionization, effective p*K* were determined by following reported procedure as described in ESI†.[36] Stress-strain property of the APCN films in water swelled state, AFM and DSC analyses were performed by standard procedures (ESI†).[10,11]

Degradation of APCN gels, characterization of degraded species and drug loading and release experiments

Dried APCN gel films (0.5 g) in triplicate were placed in distilled water at pH 5 and 7.4 separately. The pH of the solutions was checked every day. The samples were placed in shaker maintained at 37 ^{o}C. After desirable time interval the samples were removed from the solution and washed with water. The samples were then freeze dried and weighed. After 35 days of degradation experiment, water contacted with APCNs was divided into three parts. The original solutions of pH 5 and 7.4 were directly subjected to Dynamic Light Scattering (DLS) measurements using a Merlvarn instrument at a scattered angle of 90^{o} and at temperature 25 ^{o}C. The incident light was the 488 nm line of a Photal argon laser GLS 3110. The pH of other parts of the solutions was adjusted to 5 and 9 by the addition of dilute HCl or NaOH and then DLS measurements were performed again. The solutions containing degraded species were freeze-dried. The obtained solid masses were then subjected to ^{1}H NMR and IR analyses. The ^{1}H NMR (Bruker, 200 MHz) spectra of the obtained masses were recorded by dissolving them in DMSO-d_6. IR analysis of the degraded mass was recorded after crashed with KBr. Critical micelle concentration (CMC) of the degraded mass was determined by fluorescence method using pyrene as hydrophobic probe at temperature 30 ^{o}C (ESI†). Release study of both hydrophobic prednisolone acetate and hydrophilic 5-fluorouracil was carried out as described in ESI†.



Scheme 1. Synthesis of APCN gels precursors

Cytocompatibilty of APCN gels and species formed by degradation

Cytocompatibility of APCNs and degraded species was determined by (i) MTT essay with HeLa cell line for 24 h using polystyrene tissue culture 96 well-plates as standard and (ii) hemocompatibity experiment using phosphate buffer solution (PBS) and Triton-X as negative and positive controls (ESI†). Cell viability and hemolysis (%) were obtained from the following equations:

$$\text{Haemolysis}(\%) = \frac{\text{Sample}_{540} - \text{Negative control}_{540}}{\text{Positive control}_{540} - \text{Negative control}_{540}} \quad (3)$$

$$\text{Cell viability} = \frac{\text{Sample}_{570}}{\text{Control}_{570}} \quad (2)$$

where Sample_{570} and Control_{570} indicate absorbance values of complex formed in MTT assay after treatment with APCN sample and polystyrene plate respectively. Sample_{540}, Negative control_{540} and Positive control_{540} indicate absorbance values of haemoglobin after treatment of blood cells with sample and controls respectively (ESI†).

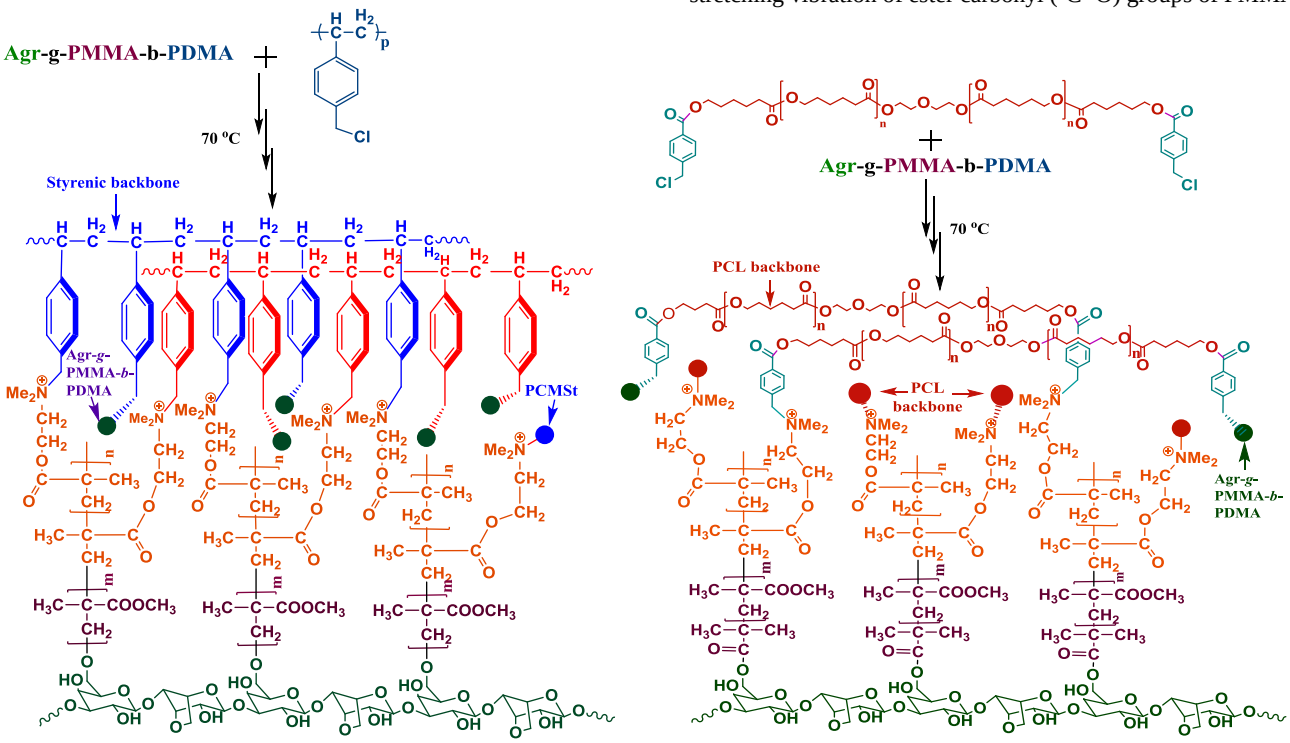
Results and Discussion

Synthesis of APCN gels

The precursors of APCN gels are the Agr-based copolymers containing attacking nucleophile (tertiary amine groups) and PCMSt or PCL containing electrophilic centre (activated chloride groups). Scheme 1 shows the synthesis of precursors of APCN gels. Table 1 Summarizes composition, M_n and PDI of Agr-based graft copolymers and a linear triblock copolymer (without Agr) used for the synthesis of APCN gels. APCN gel films were obtained by reacting PCMSt or $ClCH_2$-Ph-PCL-Ph-CH_2Cl and the copolymers (graft or linear) followed by solvent evaporation. The pendent tertiary amine moieties of the copolymers undergo sequential nucleophilic substitution reaction with phenyl activated chloride groups (Ph-CH_2-Cl) of PCMSt or $ClCH_2$-Ph-PCL-Ph-CH_2Cl (Scheme 2). The nucleophilic substitution reaction between activated chlorides and tertiary amines provided efficient cross-linking. This reaction involves no removal of leaving group and may be considered as click type of substitution reaction.

Initially, 2-10% (w/w) PCMSt was reacted with Agr-*g*-PMMA-*b*-PDMA-1 or PDMA-PMMA-*b*-PDMA-1 to evaluate the required minimum amount of PCMSt for efficient cross-linking. A 5% (w/w) PCMSt gave DMF extractable (E) as low as ca. 6-7% (Fig. S6, ESI†). Hence, 5% (w/w) PCMSt or mol equivalent of $ClCH_2$-Ph-PCL-Ph-CH_2Cl was employed for the synthesis of APCN gels. $\text{APCN}_{\text{triblock-1a}}$, $\text{APCN}_{\text{graft-1,}}$ $\text{APCN}_{\text{graft-2}}$ and $\text{APCN}_{\text{graft-3a}}$ were synthesized by cross-linking of corresponding copolymers (Table 1, entries 1, 3, 4 and 5 respectively) by reacting with PCMSt (5%, w/w). $\text{APCN}_{\text{triblock-1b}}$ and $\text{APCN}_{\text{graft-3b}}$ were synthesized by reacting $ClCH_2$-Ph-PCL-Ph-CH_2Cl (32%, w/w, i.e. mol equivalent of PCMSt) with corresponding copolymers (Table 1, entries 2 and 6 respectively). Low extractable is attributed to the facile nucleophilic substitution reaction between pendent tertiary amine groups of copolymers and activated benzyl chloride groups of PCMSt or PCL chain. Liquid nitrogen freezed APCN films were milled mechanically to obtain fine particles which were filtered through 160 μ mesh sieves. These water swelled particles are soft and injectable through hypodermic syringe of needle size 20-G. The injection of hydrogel through syringe of needle size 18-G has been reported in the literature.[37]

Representative $\text{APCN}_{\text{triblock-1a}}$, $\text{APCN}_{\text{triblock-1b}}$, $\text{APCN}_{\text{graft-3a}}$ and $\text{APCN}_{\text{graft-3b}}$ were subjected to IR and solid state ^{13}C NMR analyses. $\text{APCN}_{\text{graft-3a}}$ and $\text{APCN}_{\text{graft-3b}}$ show IR band at 930 cm^{-1} due to stretching vibration of the C-O-C bridge of 3,6-anhydrogalactose of Agr (Fig. 1A). This band is absent in $\text{APCN}_{\text{triblock-1a}}$ and $\text{APCN}_{\text{triblock-1b}}$. The band at 1730 cm^{-1} for APCN gels is due to stretching vibration of ester carbonyl (-C=O) groups of PMMA,



Scheme 2. Probable structure of APCN gels formed by combining graft copolymer and PCMSt (left) or $ClCH_2$-Ph-PCL-Ph-CH_2Cl (right).

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PDMA and PCL, depending on types of gel. The IR spectra of Agr-I and precursors of APCNs also show such characteristic bands (Fig. S4, in ESI†). The IR intensity of -C=O stretching band of $APCN_{triblock-1b}$ and $APCN_{graft-3b}$ increased compared to other APCNs owing to appearance of additional -C=O stretching vibration of the ester carbonyl group of PCL chains. Absence of band at ca. 685 cm^{-1} ($-CH_2$-Cl attached to phenyl ring) in the IR spectra of APCNs indicates near complete substitution reaction through nucleophilic attack at $-CH_2$-Cl by tertiary amine of PDMA groups. This is due to the much higher concentration of DMA (0.13-0.44%, mol/mol) unit compared to CMSt or $ClCH_2$-Ph-PCL-Ph-CH_2Cl (ca. 0.033%, mol/mol) used for the cross-linking reaction. Hence, assuming near complete reaction, equivalent of DMA units to that of activated chloride units becomes quaternized (Schemes 1 and 2). This is reasonable since, the reaction between tertiary amine and activated alkyl halide is fast and facile in DMF which lowers the probability of existence of unreacted halide group in presence of excess tertiary amine moieties. Fig. 1B shows the solid state ^{13}C NMR spectra of the APCNs. Characteristic signal (δ =46.5 ppm) of methyl chloride ($C_6H_5\underline{C}H_2$-Cl) is absent in the spectra which further implies near complete reaction of chloromethyl groups. The signals (a-d) at δ values 16.46, 45.1, 52.1 and 57.5 respectively are for methyl carbon ($-CH_3$), quaternary carbon (-C-), methoxy carbon ($-O-CH_3$), and methylene carbon ($-CH_2$-) of PMMA and PDMA. The signal (k) at ca. 180 ppm is due to –C=O of ester groups of both PMMA and PDMA. The spectra of $APCN_{graft-3a}$ and $APCN_{graft-3b}$ show additional signals (signals f and g) at δ values 70-80 ppm and signal (h) near 100 ppm due to various carbons of Agr backbone. Low intensity peaks at 125-140 ppm may be ascribed to the aromatic carbon (from halide cross-linkers).

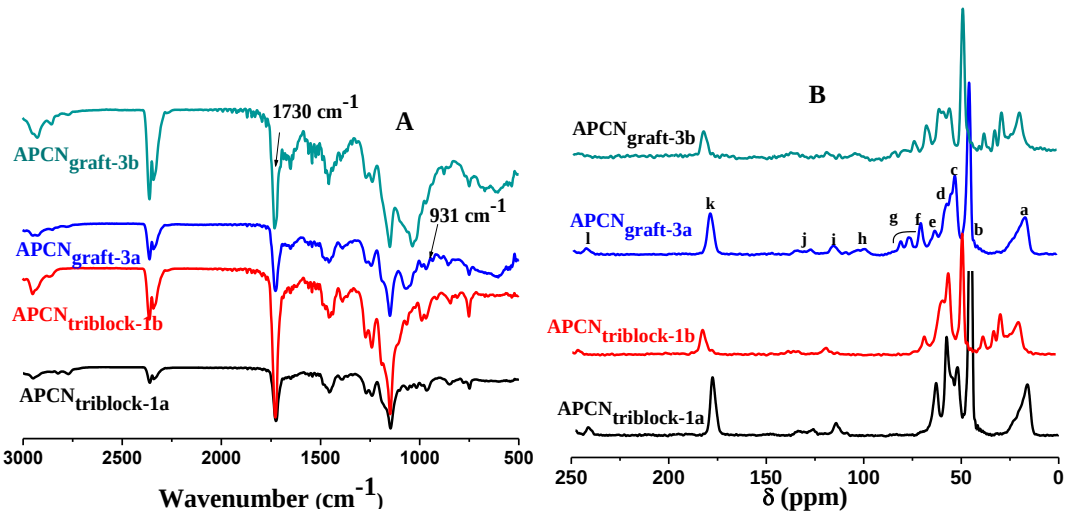

Fig. 1 IR (A) and solid state ^{13}C NMR (B) spectra of representative $APCN_{triblock-1a}$, $APCN_{triblock-1b}$, $APCN_{graft3a}$ and $APCN_{graft-3b}$.

Table 2. Composition, extractable (E) and equilibrium swelling of APCN gels.

APCN	[a]APCN Composition (%, w/w)				E (%)	Swelling (%)	
	Agr	PDMA	PMMA	PCMSt/PCL		Water (S_w)	Toluene (S_t)
$APCN_{triblock-1a}$	0	65	29	6/0	7.1 (±1)	61 (±3)	58 (±3)
$APCN_{triblock-1b}$	0	45	20	0/35	9.7 (±2)	19 (±3)	185 (±6)
$APCN_{graft-1}$	56	19	19	6/0	6.4 (±1)	49 (±2)	34 (±4)
$APCN_{graft-2}$	30	37	27	6/0	7.3 (±2)	42 (±1)	46 (±3)
$APCN_{graft-3a}$	56	25	13	6/0	7.2 (±2)	128 (±2)	35 (±3)
$APCN_{graft-3b}$	38	17	10	0/35	8.5 (±1)	102 (±3)	112 (±4)

a-from copolymer composition (gravimetric analysis) by subtracting extractable value

Extractable, pH dependent swelling and p*K*s of the APCN gels

Table 2 summarizes actual composition of APCN gels, E in DMF and swelling in water (S_w) at pH 7.4 and in toluene (S_t) for different gel films. The extent of S_w increases with increasing amount of water soluble Agr and PDMA in the APCNs. For example, $APCN_{graft\text{-}3a}$ showed highest S_w (128%) at pH 7.4 due its highest Agr+PDMA content (81%, w/w). The lowest swelling (19%) of $APCN_{triblock\text{-}1b}$ is attributed to its highest content (55%, w/w) of total PMMA and PCL. The S_t of APCN gels increases with decreasing Agr and increasing PMMA, PDMA and PCL content. This is because Agr part swells feebly in toluene whereas PMMA, PDMA and PCL attached to network swell significantly in toluene.

The degree of water swelling and pK values of five different APCNs have been determined (Fig. S7, ESI†). Degree of swelling increases with lowering of pH and vice versa. This is simply due to enhanced protonation of DMA moieties and electrostatic repulsion caused by the protonated DMA moieties in the network with lowering of pH.[36] Such pH responsive swelling is important which influences the release kinetics of drugs from the APCNs (vide infra).

The p*K* values of the APCNs were determined from the degree of ionization vs pH plots (Fig. S7, ESI†).[36] The p*K* values follow the order for the APCNs, $APCN_{graft\text{-}3a}$> $APCN_{graft\text{-}3b}$> $APCN_{graft\text{-}1}$> $APCN_{triblock\text{-}1a}$~$APCN_{graft\text{-}2}$. This is because the extent of water swelling of the APCNs follows the similar trend but the amount of relatively hydrophobic part in the APCN gels follows the opposite trend (Table 2). As a result, water diffusion through the APCNs renders the ionization easier for APCN gel having high degree of water swelling. It was reported that the architecture of network does not influence the degree of ionization.[36] Both $APCN_{triblock\text{-}1a}$ and $APCN_{graft\text{-}2}$ exhibited close p*K* values (p*K*=4.7-4.8) due to presence of similar amount of PMMA (ca. 27-29%, w/w) in the respective APCNs (Fig. S7, ESI†). This implies no effect of APCN gels architecture on degree of ionization.

Tensile stress-strain property and phase separation behaviour of the APCN gels

The tensile stress-strain behaviour of APCN gels was significantly influenced by their degree of water swelling (Fig. 2). The tensile stress (at break) follows the order for the APCNs, $APCN_{triblock\text{-}1b}$> $APCN_{graft\text{-}1}$>$APCN_{triblock\text{-}1a}$> $APCN_{graft\text{-}3b}$~$APCN_{graft\text{-}3a}$. This order of tensile stress is due to enhanced swelling of the APCNs in opposite order.

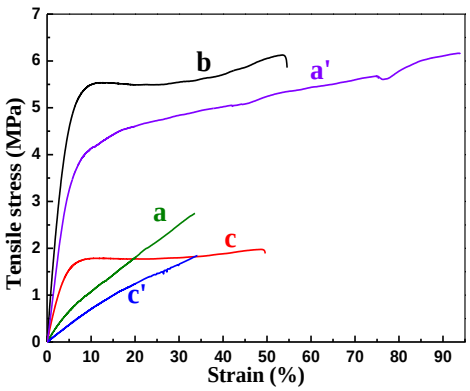

Fig. 2 Stress-strain profiles of (a) $APCN_{triblock\text{-}1a}$, (a') $APCN_{triblock\text{-}1b}$ (b) $APCN_{graft\text{-}1}$, (c) $APCN_{graft\text{-}3a}$ and (c') $APCN_{graft\text{-}3b}$. Stress-strain measurements (up to failure) were performed with the water swelled films.

Transparency of the APCN films in both dry (Fig. 3, pictures A and G) and water swelled states (Fig. 3, photographs B and H) indicates co-continuous nanophase morphology. The co-continuous morphology of the APCN films was also evident by the homogeneous distribution of negatively charged RB dye and neutral riboflavin. Clearly, RB (Fig. 3, photographs C, D, I and J) and riboflavin (Fig. 3, photographs E, F, K and L) adsorbed films in their dry and water swelled states show high degree of transparency. Since, APCNs are optically transparent; the domain size must be smaller than the wavelength of light.

The DSC thermograms (Fig. 4, thermograms b and c) of $APCN_{graft\text{-}3a}$ and $APCN_{graft\text{-}1}$ show two T_g at 32 °C and 91 °C due to PDMA and PMMA parts of the copolymer. The T_g of $APCN_{triblock\text{-}1a}$ also appears at 32 °C. These T_g values indicate some degree of phase miscibility between PDMA and PMMA. This is because the mechanical blend of PMMA and PDMA showed two T_g corresponding to the T_gs of PMMA (T_g= ca. 100 °C) and PDMA (T_g= ca. 35 °C).[38] On the other hand, $APCN_{graft\text{-}3b}$ and $APCN_{triblock\text{-}1b}$ show single T_g at ca. 68 °C due to mixed PDMA/PMMA part. This indicates relatively high degree of phase miscibility in PCL containing APCNs.

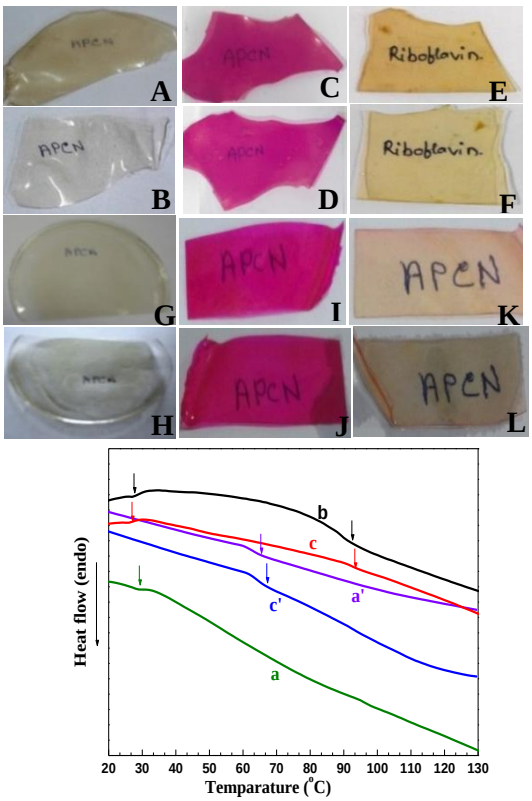

Fig. 3 Digital photographs of (A) dry, (B) water wet, (C) dry RB adsorbed, (D) water wet RB adsorbed, (E) dry riboflavin adsorbed and (F) water wet riboflavin adsorbed $APCN_{graft\text{-}3a}$ showing transparency. Photographs G to L are the corresponding pictures of $APCN_{graft\text{-}3b}$. Plots: DSC profiles: (a) $APCN_{triblock\text{-}1a}$, (a') $APCN_{triblock\text{-}1b}$, (b) $APCN_{graft\text{-}1}$, (c) $APCN_{graft\text{-}3a}$ and (c') $APCN_{graft\text{-}3b}$.

Phase mode AFM images (Fig. 4, images A-D) of $APCN_{triblock\text{-}1a}$, $APCN_{graft\text{-}3a}$, $APCN_{triblock\text{-}1b}$ and $APCN_{graft\text{-}3b}$ also show nanophase morphology. Appearance of brighter domains in the phase images of $APCN_{triblock\text{-}1a}$ and $APCN_{graft\text{-}3a}$ may be due to the nanophase separation of relatively hard (high T_g) Agr and PMMA and the darker regions is due to the relatively softer PDMA. The relatively darker regions in the phase images of $APCN_{triblock\text{-}1b}$ and $APCN_{graft\text{-}3b}$ appear probably due to mixed PDMA (T_g= 35 °C) and

PCL (T_g= ca. -60 ^oC). The degree of phase separation is lower in $APCN_{triblock\text{-}1b}$ and $APCN_{graft\text{-}3b}$ compared to $APCN_{triblock\text{-}1a}$ and $APCN_{graft\text{-}3a}$. This is consistent with the DSC results. This may probably be due to enhanced miscibility between PMMA and PDMA in presence of PCL. PCL contains backbone ester linkages which might have enhanced the compatibility with both PDMA and PMMA attached to Agr backbone.

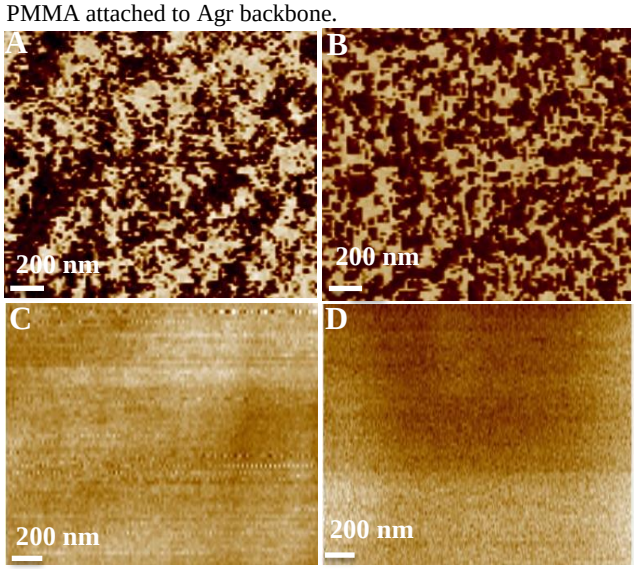

Fig. 4 Phase mode AFM images of APCN gels. Images A to D are for $APCN_{triblock\text{-}1a}$, $APCN_{graft\text{-}3a}$, $APCN_{triblock\text{-}1b}$ and $APCN_{graft\text{-}3b}$ respectively. The thin films were deposited on mica surface for AFM analysis.

Degradation of representative $APCN_{graft\text{-}3a}$ and $APCN_{graft\text{-}3b}$ and property of the degraded species

Representative $APCN_{graft\text{-}3a}$ and $APCN_{graft\text{-}3b}$ undergo ca. 24% and 21% (w/w) degradations respectively at pH 5 for up to 35 days at 37 ^oC. The degradation was ca. 12% (w/w) for both the APCNs at pH 7.4 (Fig. 5). This is attributed to (i) enhanced rate of degradation of Agr backbone and hydrolytic cleavage of ester-linkages of reacted DMA moieties at acidic pH. The IR spectra (Fig. S8, in ESI†) of the freeze-dried degraded masses are similar to that of copolymers and virgin APCNs. ^1H NMR spectra of degraded masses of the APCN gels (Fig. S9, in ESI†) indicate presence of Agr and Agr-*g*-PMMA-*b*-PDMA which are additionally grafted with methylene groups of PSt chain.

The degraded species were soluble in water and formed foam in the degraded medium owing to their surface active nature. The $APCN_{graft\text{-}3a}$ and $APCN_{graft\text{-}3b}$ exposed original solutions (pH=5) after 35 days, forms micelles with broad particle size distributions having hydrodynamic diameter ~135 nm and 142 nm respectively as confirmed by DLS analyses. Relatively narrow particle size distributed DLS curves with average hydrodynamic diameter 110 nm and 115 nm respectively were obtained when the DLS measurements were performed by adjusting the pH of the original solutions to 7.4 (Table S1). Further adjustment of pH to 9 narrowing down the distribution curve with further lowering of hydrodynamic diameter occurred (Table S1 and Fig. S10, in ESI†). These results indicate pH responsive behaviour of the formed micelles. Degradation experiment conducted at pH 7.4 also exhibited similar DLS curves (Figure not shown).

The CMC (Table S1) of the freeze-dried masses were obtained by fluorescence experiments with pyrene as hydrophobic probe (Fig. S11, ESI†). The CMC values of the degraded species were in the range 0.12-0.14 at pH 7.4. The decrease of I_1/I_3 values with increasing concentration of degraded masses indicates pyrene entrapment ability of the formed micelles.

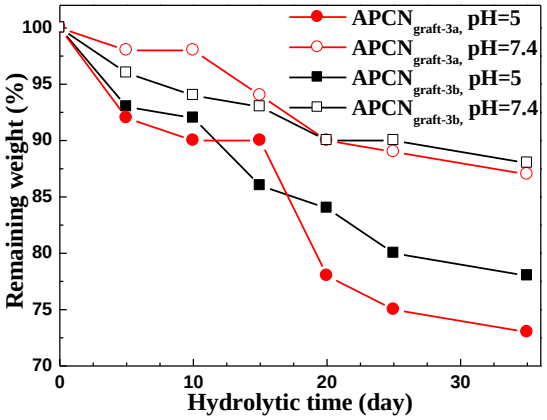

Fig. 5 Degradation profiles of representative $APCN_{graft\text{-}3a}$ and $APCN_{graft\text{-}3b}$ at pH 5 and 7.4 respectively.

Drug release from the APCN gels

The release behaviour of a model hydrophobic prednisolone acetate and hydrophilic 5-fluorouracil was studied by incorporating into APCN gels. The experimental setup was designed in such a way to obtain the released drug from the gels in the environmental water (separated by semipermeable membrane) which is free from species formed by degradation of APCN gels. The release medium (outside the dialysis tube) was changed with fresh one after certain time while the release medium inside the tube was fixed. The APCN gels (except $APCN_{triblock\text{-}1a}$) degraded with time and thus the water inside the dialysis membrane contains increasing amount of degraded species with time in the form of micelles. This experiment was performed to confirm barrier if any imposed by the formed micelles. The release rate of hydrophobic prednisolone acetate or hydrophilic 5-fluorouracil was higher at pH 5 than that at pH 7.4 from the APCNs due to greater degree of swelling and enhanced rate of degradation of the APCNs at pH 5 (Fig. 6). The sustained release profiles of prednisolone acetate indicate slow diffusion controlled release. The rate of prednisolone acetate release was slower with $APCN_{graft\text{-}3b}$ and $APCN_{triblock\text{-}1b}$ (Fig. 6B) compared to corresponding $APCN_{graft\text{-}3a}$ and $APCN_{triblock\text{-}1a}$ respectively (Fig. 6A) which is probably due to high degree of phase mixing in the former APCN as confirmed by AFM and DSC analyses and also due to higher amount of hydrophobic polymers (PCL and PMMA) content. High degree of phase mixing and higher hydrophobic polymer contents provide greater spreading of hydrophobic phase and lowers the water swelling which enhance the solubility of hydrophobic drug. The release of prednisolone acetate was usually slower in $APCN_{triblock\text{-}1a}$ and $APCN_{triblock\text{-}1b}$ compared to $APCN_{graft\text{-}3a}$ and $APCN_{graft\text{-}3b}$ owing to lower water swelling of formers APCN gels. On the other hand, burst release of 5-fluorouracil was observed from $APCN_{graft\text{-}3a}$, $APCN_{triblock\text{-}1a}$ and $APCN_{triblock\text{-}1b}$ within short release time and then release kinetics followed almost linear relationship (Fig. 6C). The typical burst release may be attributed to the release of loosely surface bound sacrificial drugs. The rate of 5-fluorouracil release was also depends on water swelling of APCN gels as was observed for prednisolone acetate.

The concentration of drugs (g/L) inside (fixed release medium) and outside (fresh release medium) the dialysis membrane was similar up to ca. 70 h of release and then the concentration of drugs increases inside the dialysis membrane. For example,

concentrations of released prednisolone acetate by $APCN_{graft\text{-}3a}$ were 237±4 mg/L and 170±3 mg/L in the inside and outside the dialysis tube respectively after 350 h of release at pH 7.4. Similarly, concentrations of released prednisolone acetate from $APCN_{graft\text{-}3b}$ were 210±6 mg/L and 110±4 mg/L inside and outside the dialysis tube respectively after 350 h of release at pH 7.4. This phenomenon strongly indicates extra barrier caused by polymer micelles which are formed after (70-75 h) sufficient degradation (above CMC) of APCN gels. The solution (inside dialysis membrane) containing micelles and prednisolone acetate after 350 h of release study also exhibited pH dependent delayed release of drug (S12, ESI†).

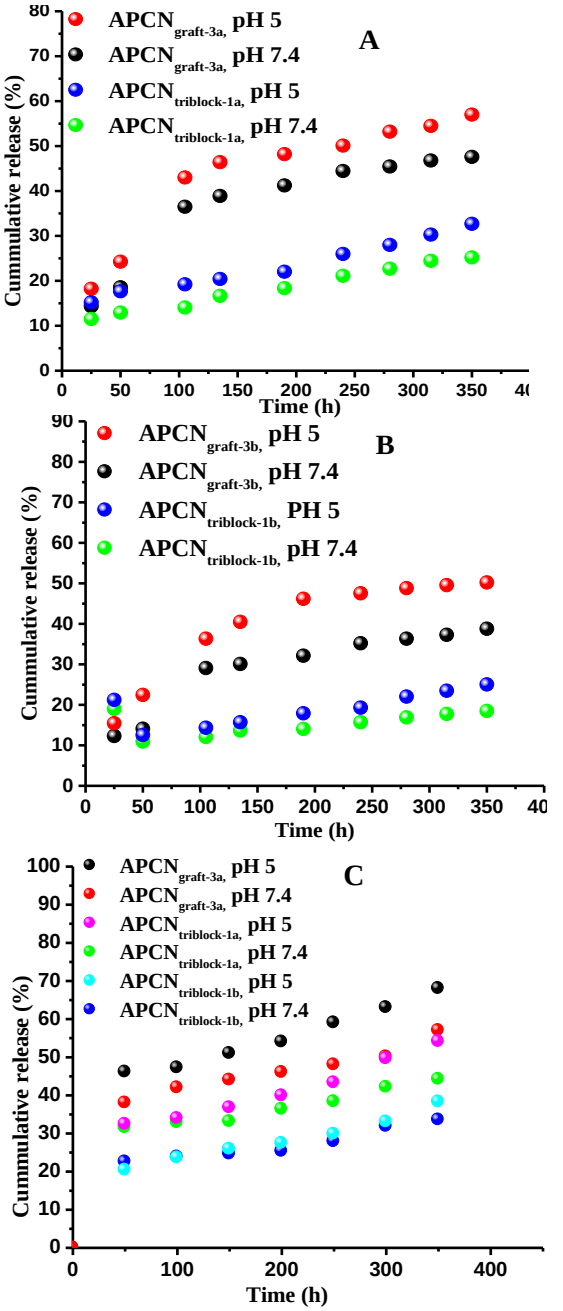

Fig. 6 Cumulative release of prednisolone acetate from $APCN_{graft\text{-}3a}$ and $APCN_{triblock\text{-}1a}$ (A), $APCN_{graft\text{-}3b}$ and $APCN_{triblock\text{-}1b}$ (B) and cumulative release of 5-fluorouracil from $APCN_{graft\text{-}3a}$, $APCN_{triblock\text{-}1a}$ and $APCN_{triblock\text{-}1b}$ (C) at outside the dialysis tubes at pH 5 and 7.4 at 37 °C.

Cytocompatibilty and hemocompatibility

Cytocompatibility of APCN gels and species formed by degradation of representative APCN gel was determined by MTT assay using HeLa cell line (Fig. 7A). The MTT assay indicates high degree of cell viability after treatment HeLa cells with various APCN gels. The species formed by degradation of representative $APCN_{graft\text{-}3a}$ also exhibited high degree of cytocompatibilty. The high degree of cell viability and obviously no loss in cell viability compared to cell treated with polystyrene tissue culture plate (standard) indicates cytocompatibility of APCN gels and degraded species of APCN gel.

Hemocompatibility is also an essential criterion for use of biomaterials in contact with blood. Hemoglobin released from red blood cells is inversely proportional to the hemocompatibility of the gel materials. Hemolysis value for hemocompatibility is in the range 10 to 25%.[39] Generally, hemolysis less than 20% is considered to be hemocompatible. Hemocompatibility of our APCN gels and degraded species of representative $APCN_{graft\text{-}3a}$ was also determined by treating the samples with red blood cells (5 mg samples with 100 µL RBC stock solution) (Fig. 7 B). Low degree of hemolysis (5-6%) once again indicates blood compatibility of our APCN gels. The good cytocompatibilty and blood compatibility of APCN gels and degraded species are ascribed to the compatibility of precursor polymers.

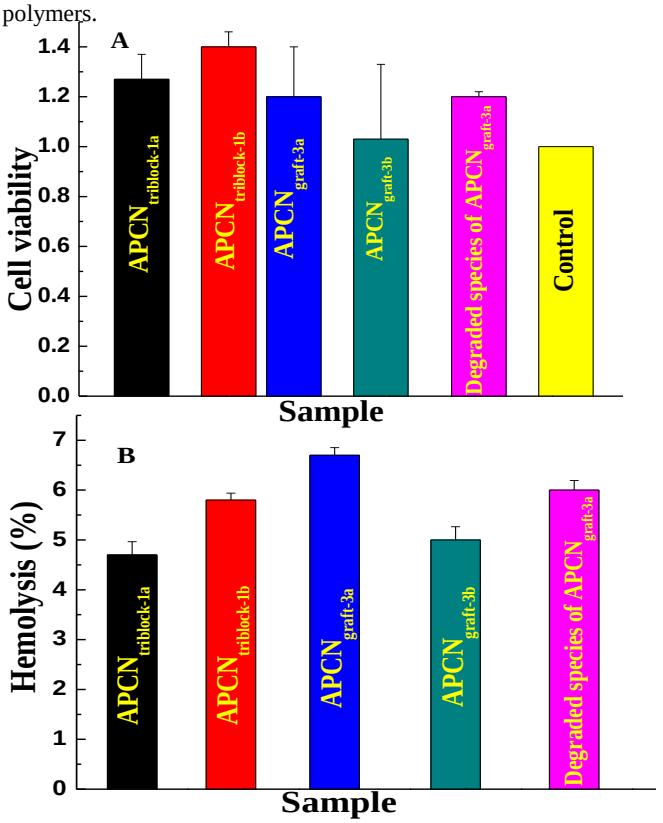

Fig. 7 Viability of HeLa cells after 24 h of incubation with APCN gels and species formed by degradation of representative $APCN_{graft\text{-}3a}$ at 37 °C (A) and hemocompatibility of APCN gels and degraded species of $APCN_{graft\text{-}3a}$ after incubation with blood cells for 1h at 37 °C (B). The viability was measured by the standard MTT assay and was expressed as a relative to cells grown in the presence of polystyrene standard. In all experiments: n=3 and standard deviation is shown as bar.

Conclusions

Synthesis of degradable/cytocompatible and pH responsive APCN gels has been successfully accomplished by click type of sequential nucleophilic substitution reaction between tertiary amine groups of agarose-based graft copolymers or fully methacrylate-based triblock copolymer and activated benzyl chloride groups of polycaprolactone or poly(chloromethyl styrene). This reaction avoids the formation of by product due to quaternization of nitrogen atoms. Transparency of the APCN gel films in both dry and swelled states, dye entrapment study, DSC and AFM analyses confirmed co-continuous nanophase separated morphology. The degree of phase separation is lower in PCL containing APCNs compared to PCMSt containing APCNs. These APCN gels undergo degradation in aqueous medium and the degraded species formed micelles. The APCN gels are able to entrap and release both hydrophobic and hydrophilic drugs and exhibit pH responsive behaviour. The release kinetics also influence by phase separation behaviour of the APCNs and extent of their water swelling. The release of drug by the APCNs (except $APCN_{triblock\text{-}1a}$) experiences double barrier after certain time of release experiments due to additional barrier provided by generated micelles. The milled water swelled soft particles of APCN gels are injectable through hypodermic syringe of needle size 20-G and may be suitable for controlled release application. Preliminary experiments indicate that the APCNs and degraded species exhibit good cytocompatibility and hemo compatibility. It is possible to change the composition, structure and architecture of the copolymers for the synthesis of APCNs for further application. Thus, the synthetic approach is versatile and there is further opportunity for synthesis of library of APCN gels and selection thereof for particular application.

Conflicts of interest

The authors declare no competing financial interest.

†Electronic supplementary information (ESI) available. See DOI:xxx

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

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Amphiphilic conetwork gels based on graft copolymers of agarose and polycaprolactone exhibited desarable cytocompatibility/blood compatibilty, pH resonsive release of hydrophilic and hydrophobic drugs and may be sutable for biomedical applications.