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ORIGINAL CONTRIBUTION

Three-dimensional porous HPMA-co-DMAEM hydrogels for biomedical application

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Abstract The aim of this work is to develop a novel biocompatible drug delivery carrier and tissue engineering scaffold with the ability of controlled drug release and also tissue regeneration. We have synthesized *N*-(2-hydroxypropyl)methacrylamide and 2-(dimethylamino)ethyl methacrylate copolymer-based hydrogels loaded with doxorubicin and tested in vitro. The manifestation of temperature sensitivity is noted with a sharp decrease or increase in hydrogel optical transparency that happens with the temperature exceeding a critical transition value. The drug release profile exhibited pH-sensitive behavior of the hydrogel. The hydrolytic degradation of gel and in vitro studies of polymer–doxorubicin conjugate and doxorubicin release from hydrogel matrix indicated that hydrogels were stable under acidic conditions (in buffers at pH 4.64 and 6.65). In both drug forms, polymer–doxorubicin conjugate and free doxorubicin could be released from the hydrogel scaffold at a rate depending directly on either the rate of drug diffusion from the hydrogel or rate of hydrogel degradation or at rate controlled by a combination of the both processes. In vitro analysis showed homogenous cell attachment and proliferation on synthesized hydrogel matrix. In vivo implantation demonstrated integration of the gel with the surrounding tissue of mice within 2 weeks and prominent neo-angiogenesis observed in the following weeks. This multifunctional hydrogels can easily overcome biological hurdles in the in vivo conditions where the pH range changes drastically and could attain higher site-specific drug delivery improving the efficacy of the treatment in various therapeutical applications, especially in cancer therapy, and could also be used as tissue engineering scaffold due to its porous interconnected and biocompatible behavior.

Keywords Cancer therapy · Dual responsive · Drug delivery system · In vivo bio-compatibility · Tissue engineering

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Introduction

The quest for site-specific drug delivery system has led to the advancement in fabrication of highly evolved biomaterials with improved biocompatibility and biodegradability properties. In recent years, extensive focus has been devoted to fabricating "intelligent" or "smart" hydrogels that respond rapidly to any external stimuli changes along with biocompatibility and high water-absorbing capacity [1–5]. These hydrogels are synthesized to respond to external stimulus change by swelling and deswelling. Polymeric hydrogels that exhibit a reversible change of its property in response to external stimuli like temperature, pH, ionic strength, and analyte concentration are favorable materials for use in controlled drug delivery systems [5–9], chemical sensors [10], "intelligent" windows [11], and numerous other biomedical applications [12]. Several groups have extensively searched for polymer either in hydrogel forms or solution that responds to both temperature and pH [13–15]. Hydroxypropyl methacrylate is an attractive candidate as the hydrophilic block of polymeric material [16]. Besides being biocompatible and nonimmunogenic, it also exhibits physicochemical properties that mimic natural living tissue [17–19]. *N*-(2-hydroxypropyl)methacrylamide (HPMA) has one major advantage over PEG, that is, its multifunctionality provides site for multiple targeting molecules or drug or proteins to be conjugated onto the same

polymer chain [20–22]. It also shows hydrolytic and chemical stability along with being favorable to cells entrapped in the polymeric capsule (Horia), making it one of the most extensively used materials in tissue engineering and drug delivery system.

A new hydrophilic, biocompatible polymer, based on HPMA [23, 24], is chosen as a matrix for polymeric drug carrier. Due to the α-carbon substitution, the N-substituted amide bond ensures hydrolytic stability of the side chains [25]. In addition, the crystallinity of the monomer guarantees the absence of divinyl compounds, a problem with hydrophilic esters of HPMA, and the linearity of the macromolecules. The polymer chains are in close proximity and interact with each other as a fluid enters the hydrogel, resulting in the polymer chains undergoing hydration and other interactions such as hydrophobic or electrostatic interactions [26–28]. Under appropriate conditions, the hydrogel reaches a state in which the polymer chains are fully extended, and only the cross-linkers prevent the material from dissolution [29, 30].

Hydrogels based on HPMA is synthesized in order to prolong drug bioavailability and reduce side effects of free drug availability. HPMA hydrogels offer an attractive advantage over other available system as these gels are not required to be removed from the site of implantation after the depletion of drugs as the hydrolytically cleaved gels degrade into polymeric fragments with MW lower than renal excretion threshold (40 kDa). In addition, the use of HPMA as a basic monomer for hydrogel synthesis has a practical benefit since the essential products of biodegradation are soluble HPMA polymers [13, 16, 30–34]. Another important aspect of using HPMA and 2-(dimethylamino)ethyl methacrylate (DMAEM) is that the temperature-responsive hydrogel could be easily fabricated as the acrylamide pendent and dimethylamino ethyl group attribute to the hydrophobic interaction, thereby exhibiting lower critical solution temperature (LCST) resulting in dual-responsive behavior of the gels.

In this study, we have fabricated pH-sensitive hydrogels which are a copolymer of HPMA and 2-(dimethylamino)ethyl methacrylate using polypropylene glycol dimethacrylate (PPGDMA) as the cross-linking agent. The tertiary amine groups on DMAEM are weakly basic but attain a charge at low pH values, resulting in the swelling of the hydrogel. The transition pH value for DMAEM homopolymers is approximately 9, but this can be decreased by copolymerizing DMAEM with hydrophobic monomers of HPMA, making it possible to regulate transition pH value for suitable application. The release behaviors of model drug dye and doxorubicin (DOX), using changed swelling behaviors by pH value, were further evaluated. The swelling ratio of the hydrogels was evaluated by varying pHs, and the loaded drug in hydrogels is released into body simulating fluid. General phenomena of the release of drug loaded on the matrix are either by diffusion or degradation.

Materials and methods

Materials

Hydroxypropyl methacrylate, 2-(dimethylamino)ethyl methacrylate, poly(propylene glycol) dimethacrylate, ethylene glycol (EG), ammonium peroxydisulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), doxorubicin, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, streptomycin, trypsin/EDTA, and glutaraldehyde (50 %) were all purchased from Sigma-Aldrich, Yongin, Korea. Buffer solution (Fluka), acetate buffer solution (Riedel-de Haen), and phosphate-buffered saline were all bought from Sigma-Aldrich, Yongin, Korea. HeLa cells were purchased from a Korean cell bank (Seoul, South Korea).

Synthesis of hydrogels

HPMA/DMAEM hydrogels were prepared by free radical copolymerization. Different polymer concentrations for compositions for the pre-gel solution were optimized. The solution was deoxygenated by nitrogen gas after monomers were mixed at 25 °C with magnetic stirrer. Water and EG solution with TEMED was mixed as reaction activator. Finally, APS (10 % in distilled water) was added to the mixture and gently stirred for few seconds. The solution was injected into the space between two glass plates, and the reaction was allowed to proceed at 25 °C for overnight. After the incubation time, the hydrogel was detached from a glass plate and cut into a circular form (dia ~10 mm) and was rinsed to remove remaining non-cross-linked polymer, cross-linker residues in phosphate-buffered saline (PBS) buffer for 3 days at 25 °C, and buffer solutions were periodically changed.

Swelling kinetics of hydrogel dimension measurement

The hydrogels (dia ~8 mm; weight ~0.32 g) were lyophilized at −80 °C for 48 h to determine the dry weight for dimension measurement. The hydrogels were immersed in buffer solution pH 9.36, phosphate buffer pH 7.42 and 6.65, and acetate buffer pH 4.68 at body temperature 37 °C to reach the absorption equilibrium. The dry weight of the hydrogels was noted, followed by saturating the gels in buffer. The degree of swelling of the hydrated hydrogels was measured using the equation below:

$$\text{Water content}(\%) = \frac{W_s - W_d}{W_d} \times 100$$

where W_s and W_d are the weights of hydrated (wet) and dry hydrogel, respectively.

Hydrolytic degradation of the HPMA-DMAEM hydrogel

The degradation studies were performed by incubating gels at 37 °C in different pH solutions. The original weight of the hydrogels was noted, and experiment was carried on until total degradation of gel into soluble product. The track of degradation was followed as dry weight loss.

Drug release profile in vitro evaluation

Hydrogel samples were loaded with DOX, a potent anticancer drug, using its osmotic effect in order to investigate release behavior according to pH value. The absorbance of drug was measured in order to investigate amount of drug released from the hydrogels using UV (UV-2600, SHIMADZU) spectrophotometer at 490 nm. The DOX was loaded onto 12 hydrogel (diameter is 10 mm; thickness is 0.75 mm) samples, wrapped in aluminum foil, stirred in the dark with buffers, and incubated at 37 °C. The release kinetics of drug were checked at different pHs using phosphate buffer pH 7.42 and 6.65, buffer solution pH 9.36, and acetate buffer solution pH 4.68. The rate of drug release was expressed as micromoles per milliliter of a drug released in a given time period.

Biocompatibility assay

To measure the cytotoxicity and biocompatibility of the hydrogel, HPMA/DMAEM disc were separately placed in 24-well plates containing 1 ml DMEM media with NIH3T3 (seeding density 1×10^4 cell/well). Effect of DOX released by HPMA hydrogel was performed parallel to biocompatibility experiment. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a spectrophotometeric analysis, was used to quantify the end product resulting from oxidation of MTT solution by mitochondria of metabolically active cells and is regarded as an indirect assay for evaluating the rate of cell growth and proliferation. Briefly, media were gently removed from the hydrogel disc and washed by PBS (0.1 M), and then, MTT solution (0.5 %) containing thiazoyl blue, which was prepared using DMEM (serum free), was added, and plates were incubated at 37 °C for 4 h. Later, MTT solution was carefully removed from the well, and dimethyl sulfoxide (DMSO) at a ratio 1:3 with respect to MTT was added to each test well. DMSO dissolves intracellular formazan crystals which results in developing of blue-violet color end product and is spectrophotometrically read at 490 nm. At each time point, samples were fixed with glutaraldehyde (2.5 %) for 3 h and dried for scanning electron microscope analysis.

In vivo biocompatibility of the synthesized hydrogel

In vivo studies (according the ISO 10993 regulations) were carried out in eight mice. Animals were divided in four groups (n=2 mice) for observation at three different time points. All mice had free access to food and water, and similar living conditions (temperature- and humidity-controlled environment) were maintained. This study was completed with permission/approval from an ethical rights committee, Yeungnam University, South Korea. The HPMA-DMAEM hydrogels were implanted under sterile environment. The used hydrogels were sterilized in gradient ethyl alcohol (20, 40, 60, 80, and 100 %) to ensure that gels were completely sterile at the time of implantation. Mice were anesthetized by 50:50 % mixtures of purfuran and air. The implantation regions were shaven and cleaned by 70 % alcohol before the surgery, and the scaffolds were implanted subcutaneously at the back region of mice by making a horizontal incision. The length of incision was approximately 5–10 mm. After implantation of the hydrogels, incision was closed using resorbable sutures. Animals were regularly monitored for any inflammation at the site of implantation. Hydrogel-implanted mice were sacrificed after the first, fifth, and tenth days of implantation, and skin tissues at/around the site of implantation were collected for histology. The excised tissue was fixed in formaldehyde solution until further processed for histology. Tissues were dehydrated using gradient series of ethanol solutions before embedding in paraffin. Five-micrometer-thick sections were cut of tissues by microtome (HM 360). Hematoxylin and eosin, safranin, and Masson trichrome staining were performed for observing change in overall tissue morphology and tissue response to implanted material. Safranin staining was performed on excised tissue sections for screening of infiltration of mast cells at the site of implantation. For safranin staining, sections were first dehydrated in absolute alcohol, followed by 95 % alcohol for 3 min. After dehydration step, sections were incubated in Mayer HTX solution for 10 min, followed by extensive washing of section done under running tap water up to 15 min. After washing, sections were stained with 0.1 % fast green solution for 5 min. For reducing the pH, the sections were immersed in 1 % glacial acetic acid, followed by incubation with 0.1 % safranin. Finally, sections were dried in 95 % and absolute alcohol for 2 min and stained with Masson trichrome separately.

Results and discussion

HPMA-based nanocarrier of drugs is a result of systematic research carried out on hydrophilic polymers by various groups. The α-carbon substitution along with N-substituted amide bond ensures the hydrolytic stability of its side chains. We have synthesized different concentrations

of HPMA/DMAEM hydrogels, and results of physicochemical characterization along with biological behavior of these poly(HPMA) conjugates show the stimuli-responsive gels. The optical transparency of the hydrogel varied depending upon the concentration of the polymers. Different combinations of HPMA and DMAEM were prepared at the weight ratio 1:4 to 4:1 (Table 1). The difference in the properties between hydrogels H1D4 and H2D3 was observed, but these hydrogels were mechanically week and were unable to retain its shape (Fig. 1a). However, H4D1 was mechanically stable, hence used for further evaluation. The property of this hydrogels was evaluated accordingly for stimuli responsiveness to the buffer of different pH values. Hydrogels stiffen in response to pH as its swells can be attributed to the chain stiffening as a result of finite chain extensibility. The optical photographs of H3D2 hydrogel at various temperatures 25, 30, and 35 °C at fixed pH 7.42 are shown in Fig. 1b. The optical transparency varies considerably with environmental temperature. The hydrogel sample, which was transparent at lower temperature, turned white around 30 °C. The contraction of the hydrogels can be due to hydrophobic attraction between the polymer chain pendent molecules. Presence of water near hydrophobic moieties in solution and temperature-dependent hydrogen bonding [35, 36] could results in this attraction leading to the change in the physical properties of the hydrogel at temperature above 15 °C. Most of the water-soluble polymers, like poly(ethylene glycol), ethyl acrylamide, and DMA, exhibit LCSTs that are attributed to hydrophobic interactions [37, 38]. There is a well-established relationship between the LCST and turbidity of the polymer solutions [39]; however, in addition, the thermodynamic interactions between the solvent and polymer solutions which play an impetus role in hydrogel transparency transition along with fabrications technique like concentration of free-radical initiators, all contribute to the physicochemical changes of the hydrogel.

Hydrogels swelling by hydration in buffer and dimension measurement

The effect of pH on the diameter of the hydrogels which were synthesized using HPMA/DMAEM at the weight ratio 3:2 (H3D2) at 37 °C was examined (Fig. 2a). In this study, with lower pH, the diameter of hydrogels increased remarkably between pH 4.68 and 6.65. Moreover, it was observed that the hydrogel immersed at pH 6.65 solutions swelled twofold from that of the initial state and remained stable. While the hydrogel samples at physiological pH showed least swelling or even shrinking of the gels with decreased dimensions. In Fig. 2b, the initial weight of hydrogels was measured as 0.2 g, whereas the weight was found to increase seven times at acidic pH value which could be attributed to tertiary amine group on DMAEM [40]. The hydrogel at pH 7 to 9 showed insignificant swelling with no visible change in dimension of discs in comparison to hydrogels in lower pH. The change in dimension of the hydrogel due to the pH of the solution clearly indicates the pH-responsive behavior of the synthesized gels.

Swelling equilibrium of the gels is an important characteristic as it provides understanding of the polymeric network and transportation of fluid in three-dimensional systems. Dried hydrogel was weighed and placed in different pH buffer solutions, and the weight was checked every hour until there was no further change that could be noted in the weight of the gel. The amount of water-retaining capacity of hydrogels at different pHs was evaluated in Fig. 3. Similar to the dimension measurement graph, the amount of retained water of hydrogels decreased with increasing pH value. After 24 h, water retained in hydrogel was found to be over 30 times higher than that in dried hydrogel (Fig. 4). This property could be attributed to tertiary amine groups on DMA which results in hydrophobic interaction, and in swollen state of hydrogel, the water absorbed not only acts as a plasticizer to increase the intermolecular distance between the polymer chains, thereby increasing the free volume, but also offers passage ways through which model drug could be loaded. Generally, for a given base polymer material, a higher water content in the hydrogel leads to a higher permeability [40].

Model drug release behavior

A prerequisite for the targeting in vivo anticancer/antitumor activity of the hydrogel–DOX conjugates is the release of the DOX from the polymer carries [40]. The main focus of the work was to release DOX, but not during the transport

Table 1 The ratio of polymer and cross-linker used in synthesizing HPMA/DMAEM hydrogels

Polymer concentration	Composition for hydrogels (wt%)						Gelation
	HPMA	DMAEM	PPGDMA	APS	TEMED	EG	
H1D4	1	4	0.05	0.045	0.0225	0.1	Δ
H2D3	2	3	0.05	0.045	0.0225	0.1	Δ
H3D2	3	2	0.05	0.045	0.0225	0.1	o
H4D1	4	1	0.05	0.045	0.0225	0.1	oo

Δ not stable, *o* stable, *oo* too stiff

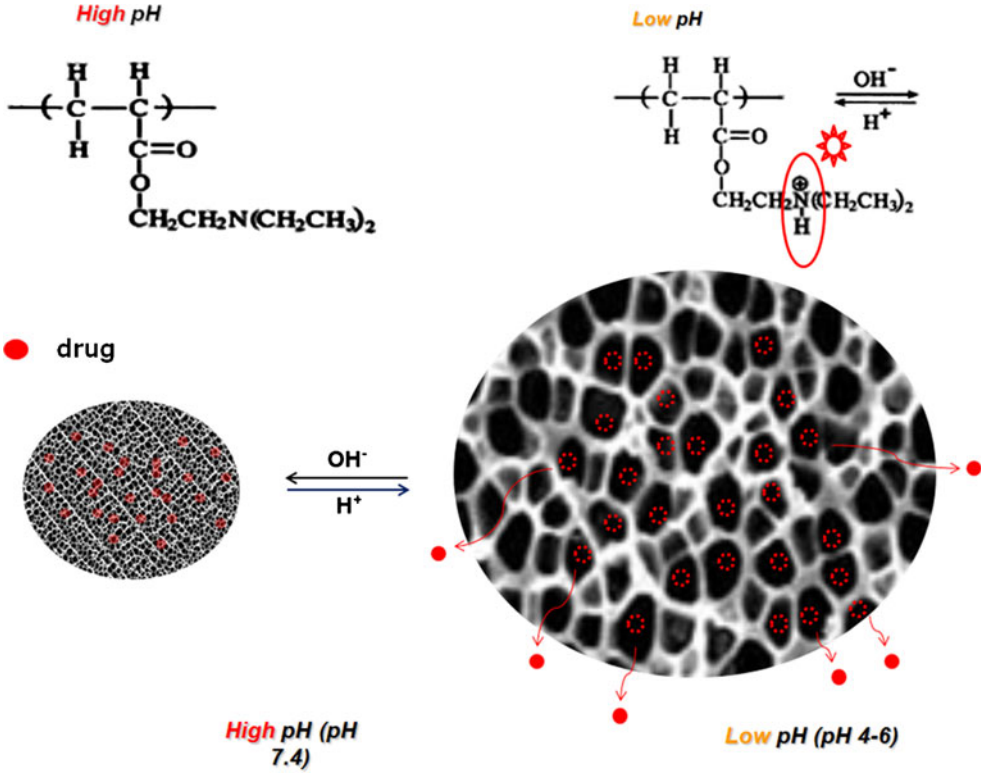

Fig. 1 Mechanism of drug release from the HPMA-DMAEM hydrogel due to swelling of the gel at lower pH

which will minimize the side effect and toxicity of the drug significantly [41]. The standard curve of DOX (anticancer) model drug was constructed by plotting UV absorbance reading of different concentrations of drug. Correlation equation obtained was utilized to establish the drug release profile which helps in determining the concentration of unknown sample. The hydrogel-loaded DOX indicated that the release was based upon hydrogel swelling ratio. The hydrogel samples were immersed for 6 days at different pH buffers at 37 °C and the DOX release profile noted at each day. The highest release rate is achieved at pH 6.65, while the lowest release rate is noted at pH 9.36. After the initial burst release at first day, both at pH 4.68 and 6.65, it could be contributed to the release of DOX load on the surface of the hydrogels. However, the sustained release of DOX by third day was noted which steadily reduced in the following time as seen in Fig. 5. The recorded absorbance is converted into the amount of drug (in micromoles per milliliter) using the standard graph. At pH 4.68 and 6.65, controlled release of drug was observed. As these hydrogels are designed for drug delivery, the pH-responsive drug release is an utmost important parameter as the pH of healthy tissue is more alkaline towards the basic, whereas pH around the tumor site is highly acidic. The HPMA-DMAEM-DOX was found to be stable at physiological pH 7.42, which was chosen to model blood environment and release of DOX at significant lower pH closer to pH of tumor and endosomes. The release of drugs at pH 6.65 can successfully be employed in achieving site-specific delivery of drugs in cancer therapies which can also increase the efficacy of treatment with precise targeting of the pathological conditions. Stimulus-responsive behavior of gels is used for fabricating smart drug delivery systems to mimic biological response in certain ways. The obvious change in pH is usually found in the in vivo conditions like GI tract which is known to be a drastic change from highly acidic pH 2 (stomach) to basic pH 5–8 (intestine); however, within different tissues too, these changes could be observed. In the chronic wounds, the pH has been reported to be around 7.4 to 5.4, depending upon the severity of infections [42, 43], and in the case of cancer tissue, the extracellular matrix is reported to have an acidic pH [44, 45]. Hence, the pH sensitivity of the matrix with slightly wider window could be a better option in comparison to the gels which swell or degrade with little change in these values. Multi- or dual-responsive systems are mostly fabricated as self-assembly micelles which react to the surrounding environment or random copolymer used for tailoring the transition point which depends upon pH or temperature; however, small change in these stimuli can result in the premature drug release. The amount of DOX release can be modulated, depending upon the detailed structure of spacers in comparison to other system such as hydrazone–DOX and aconityl conjugates [42]. HPMA–DMAEM–DOX conjugates were stable at significantly lower pH showing the stability of the system and can play a vital role in the in vivo tumor targeting. Quantitative study on HPMA/DMAEM hydrogel degradation was carried out as a function of weight loss for the

Code	HPMA	DMAEM
(a)H1D4	1	4
(b)H2D3	2	3
(c)H3D2	3	2
(d)H4D1	4	1

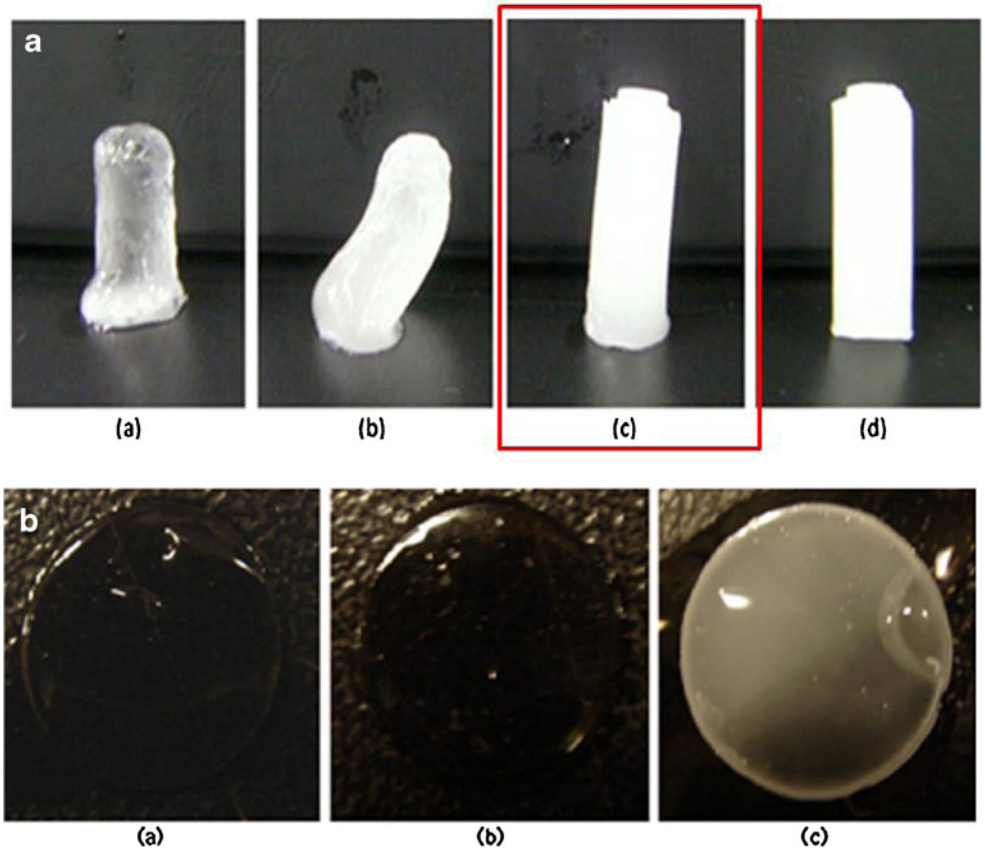

Fig. 2 Digital image of hydrogels (*A*) fabricated by varying polymer concentrations **a** 1:4 (H1D4), **b** 2:3 (H2D3), **c** 3:2 (H3D2), and **d** 4:1(H4D1). The environmental temperature was maintained at 25 °C. The changes in transparency (*B*) of the hydrogels at temperatures **a** 25 °C, **b** 30 °C, and **c** 35 °C were fixed at physiological pH 7.42

duration of incubation in different pH solutions at 37 °C as seen in Fig. 6. The degradation results and DOX release profile were found to be similar in terms of the pH-responsive behavior. The hydrogel matrix incubated in pH 6.65 showed higher rate of degradation in comparison with gel in other pH solutions. The degradation of the matrix is

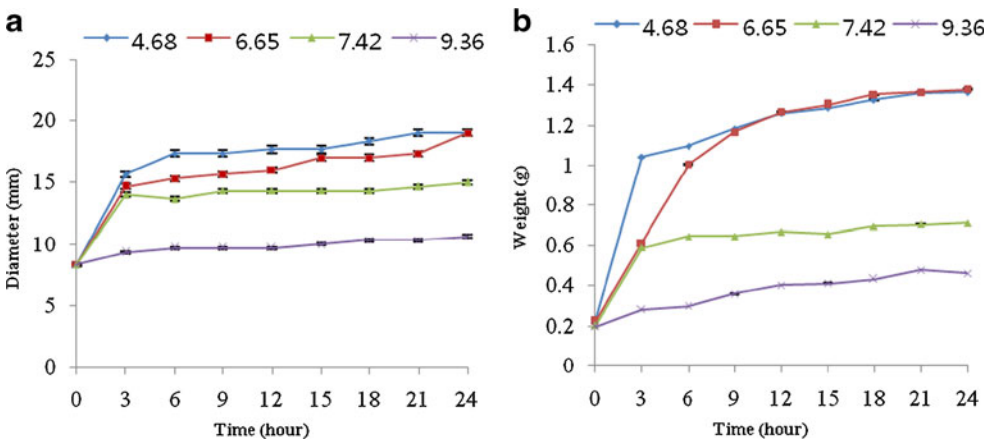

Fig. 3 Comparison of change in diameter (**a**) and weight (**b**) of HPMA/DMAEM hydrogel with time 0 to 24 h at different pHs of 4.68, 6.65, 7.42, and 9.36. The external temperature was maintained at 37 °C

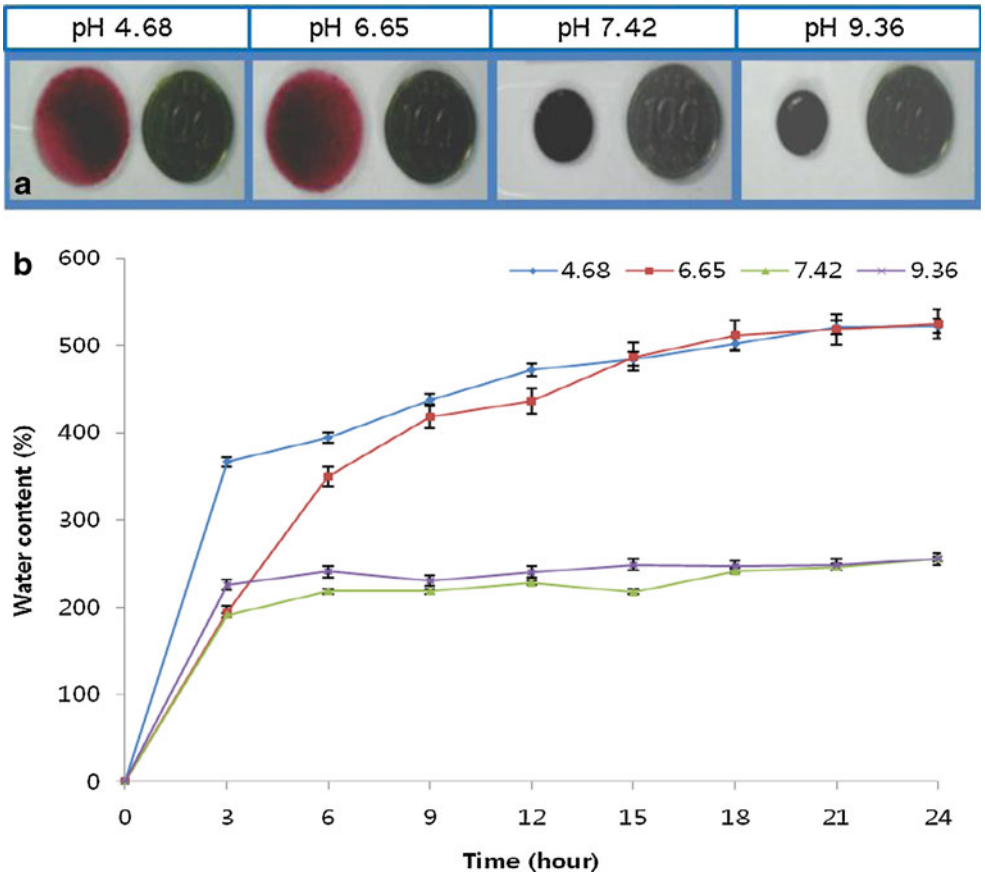

Fig. 4 Digital image of hydrogel at different pH (**a**). Comparison of change in water content (%) in HPMA/DMAEM hydrogels were calculated for 3:2 (H3D2) with time 0 to 24 h at different pH 4.68, pH 6.65, pH 7.42, pH 9.36 (**b**). The external temperature was maintained at 37 °C

an important phenomenon in the in vivo condition. The degradation needs to be in tune with drug release and/or tissue regeneration.

In vitro biocompatibility assay

The scanning electron micrograph examination revealed that HPMA/DMAEM is a porous network of the hydrogels with interconnected pores, and it has been reported that pores of scaffold contribute to the quick swelling and high water-absorbing capacity [46–49]. High porosity with interconnected polymer networks makes this and scaffold ideal for tissue engineering (Fig. 7). Cell compatibility of any polymeric material is an utmost important assay for the materials designed for an in vivo system. MTT is an indirect assay for checking the viability and metabolic activity of cells as this system provides

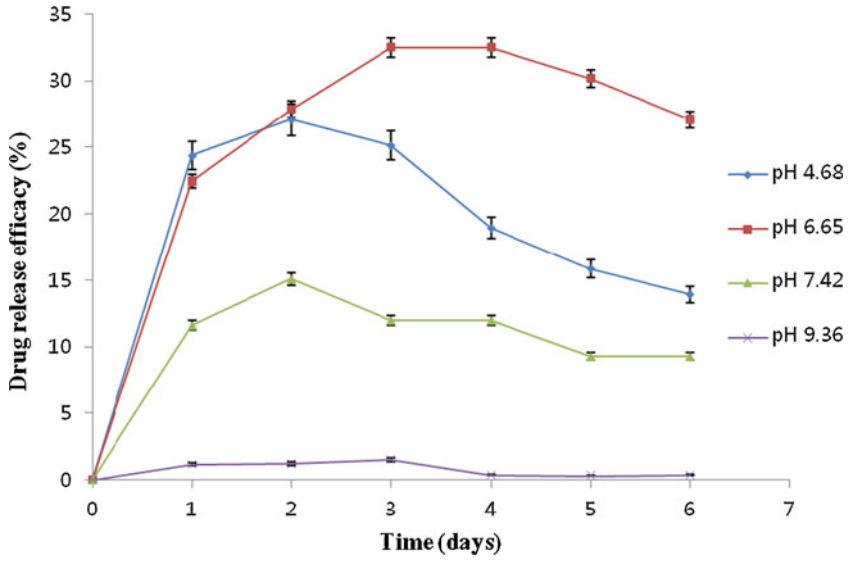

Fig. 5 The hydrogel samples were immersed for 6 days at different pHs, and the amount of DOX release at HPMA/DMAEM hydrogel was measured. The external temperature was fixed 37 °C

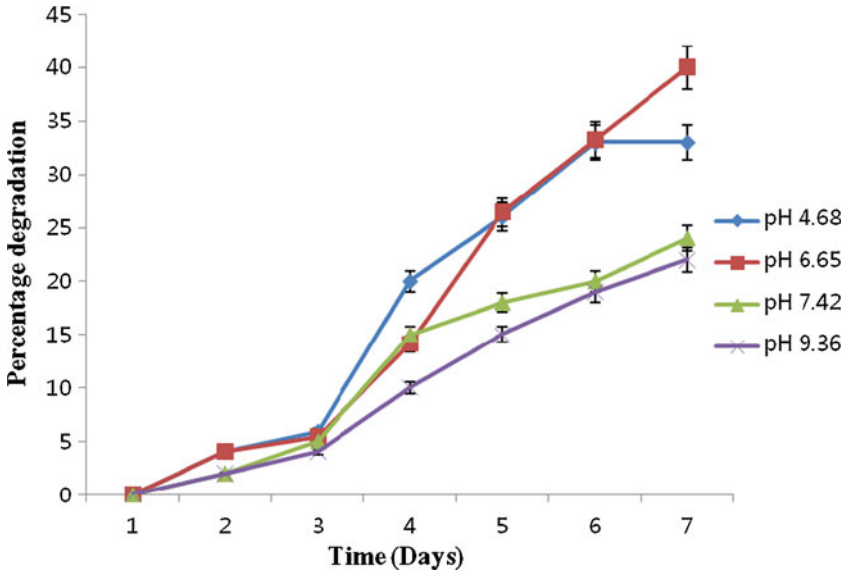

Fig. 6 Degradation (in percent) of the HPMA/DMAEM hydrogels in different pHs with respect to weight loss

reasonable idea about the cell–matrix interactions [36]. The total metabolic activity of cells was checked on hydrogel seeded with NIH3T3 sells and was found to constantly increase, indicating the hydrogel surface for use, the cell attachment and proliferation (Fig. 8). The scanning electron micrograph image clearly demonstrated the biocompatible nature of the hydrogel as cells could be seen homogenously attached and distributed throughout the matrix (Fig. 9). The cell attachment was accompanied with secretion of natural extracellular matrix (ECM) component (Fig. 9b), indicating metabolically active cells, and SEM performed on day 5 showed increased cell attachment on the matrix (Fig. 9c, d). Even on the tenth day, a high number of cells were found to be attached to the HPMA/DMAEM hydrogel matrix, indicating the biocompatible behavior (Fig. 9e, f). The scanning electron micrograph results support the MTT results as both assays confirm the biocompatibility of the material. Using DMAEM, which is not a known biocompatible material, has not hindered the cellular attachment. This result proves the potentiality of these hydrogels as an ideal matrix for tissue engineering, beside the drug delivery system as not the only drug release based on pH value of system, but these matrices were also biocompatible without eliciting adverse effect to cells.

In vivo biocompatibility of synthesized hydrogel

For tissue engineering application, HPMA/DMAEM hydrogels were implanted subcutaneously in mice for 10 days. The in vivo studies showed early angiogenesis around the implanted scaffolds. The implants were well accepted, and mice showed no signs of discomfort. In particular, no effects on sleeping or feeding habits were observed even after 6-week postimplantation period. The in vivo degradation of hydrogel was observed as the amount of material remaining which was considerably low with increase in time. Stereomicroscopic images indicated vascularization around the implanted hydrogel. New blood vessels were seen in the host tissue adjacent to the surface of the implants. No signs of adverse reactions, such as necrosis, infection, or granuloma, were observed around the implants even till the tenth

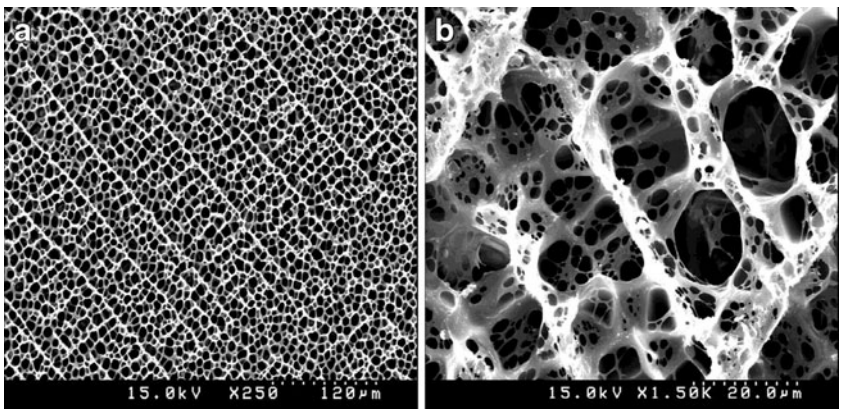

Fig. 7 Scanning electron microscope image of HPMA/DMAEM hydrogels showing homogenously distributed porous structure with interconnected pores

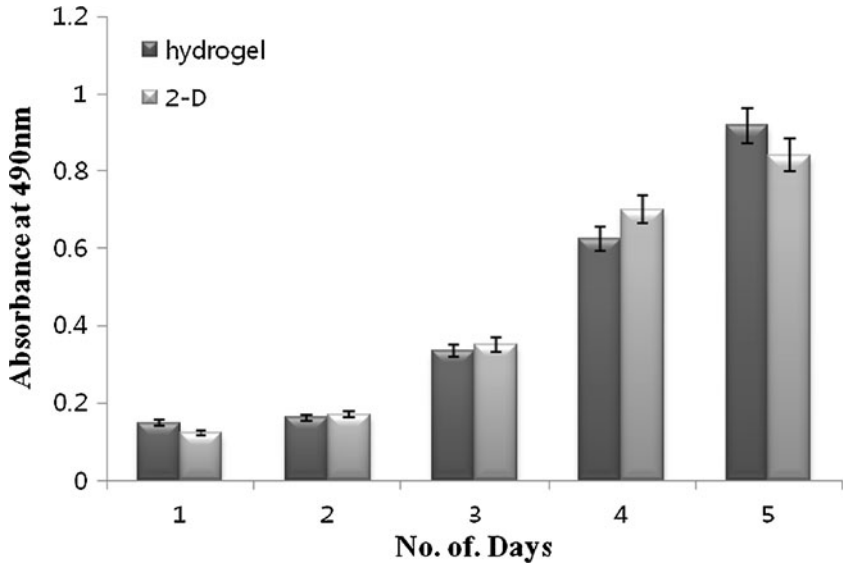

Fig. 8 MTT assay performed shows increase in the total metabolic activity of cells over period of 5 days (inset digital image of hydrogel loaded with NIH3T3 cells)

day of postimplantation. Hematoxylin and eosin staining compared with the control (Fig. 10a) showed the infiltration of cells at the site of implantation (Fig. 10b–d). A large number of cells were found at the site of implant after tenth day, demonstrating that gels can recruit surrounding cells. Masson trichrome staining of control (Fig. 10e) compared with test (Fig. 10f–h) showed the collagen fibers formation and total integration of the hydrogel with the host tissue. An enormous amount of fine blood vessel formation was observed near the implanted material in the fifth to tenth days of implantation. Furthermore, safranin staining (Fig. 10i–l) was done to check the presence of mast cells. At first 24 h, the infiltration of small volume of mast cells could be seen; however, after fifth day, there was a significant reduction in the mast cells at the site of implantation, signifying that the material does not cause any immunological response which could lead to host vs graft rejection mechanism, and scaffold was indeed recruiting surrounding cells for integration into the host tissue. The implanted hydrogel scaffolds showed mild inflammation at the site of implantation and were characterized by infiltration of macrophages, dendritic cells, and proliferation of few endothelial cells on the first day. The localized inflammation reduced with formation of thin fibrous capsule around the gel. The tissue response was

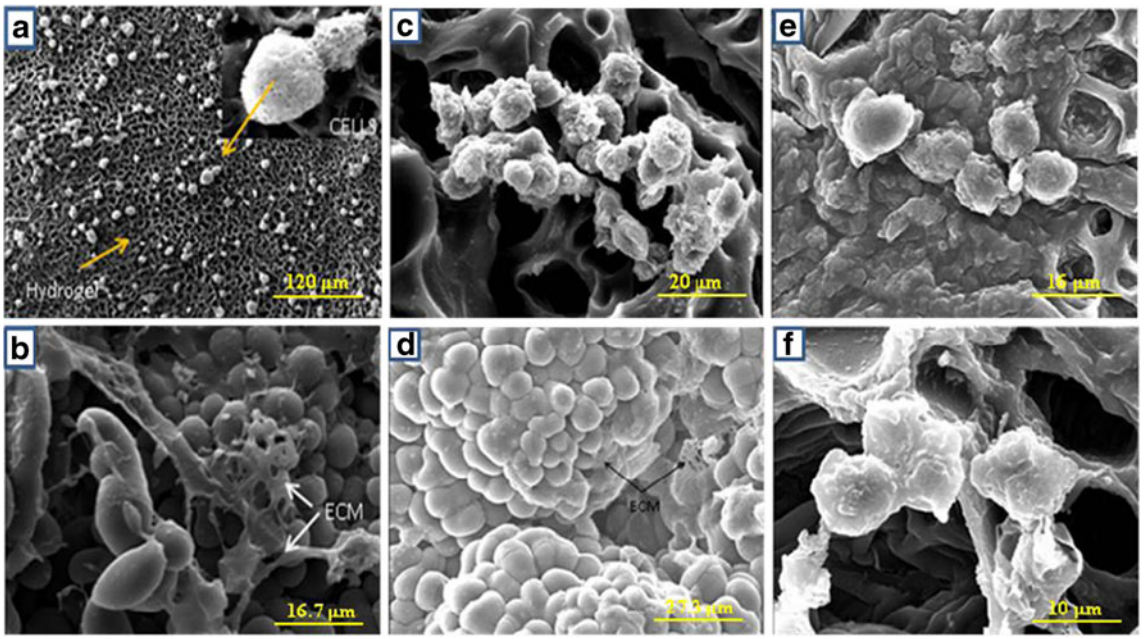

Fig. 9 Scanning electron micrograph image of HPMA/DMAEM hydrogels seeded with NIH3T3 cells. Inset cells attached to hydrogel at higher magnification (×3.00 K and scale bar 10 μm) (**a**). The cell attachment and proliferation after 24 h of seeding indicate biocompatible property of the synthesized hydrogel (**b**). Image (**c**–**f**) showing the NIH3T3 cells proliferating and secreting ECM at fifth and ninth days

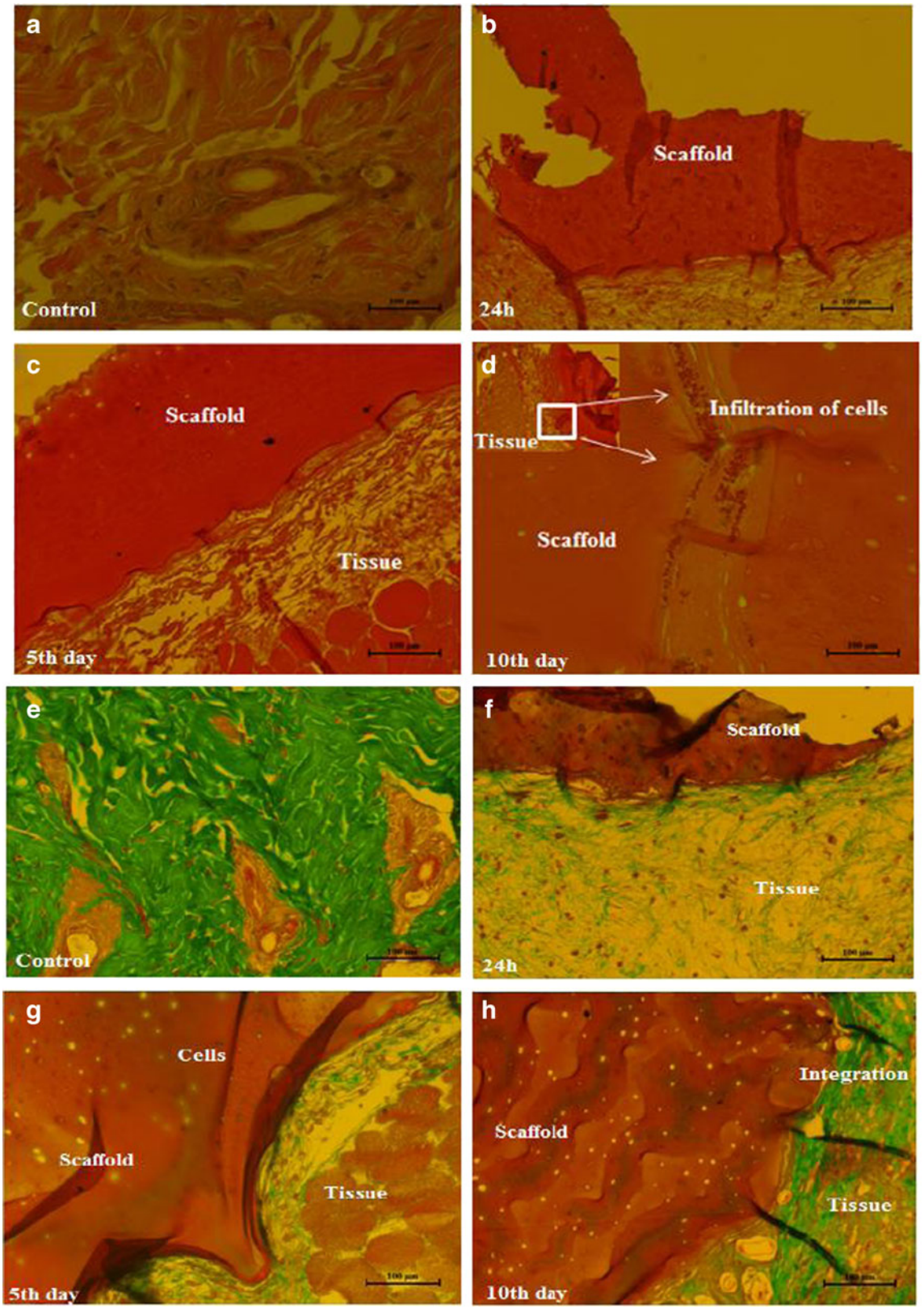

Fig. 10 Stereophotography of HPMA/DMAEM hydrogel implanted subcutaneously on mice to check in vivo biocompatibility of the gel. H & E staining (**a**–**d**). The inset image (**d**) shows the channel formed for movement of cells through the scaffold from surrounding tissue. The performed Masson trichrome (**e**–**h**) and safranin staining (**i**–**l**) showed integration of scaffold and surrounding tissue on fifth to tenth days

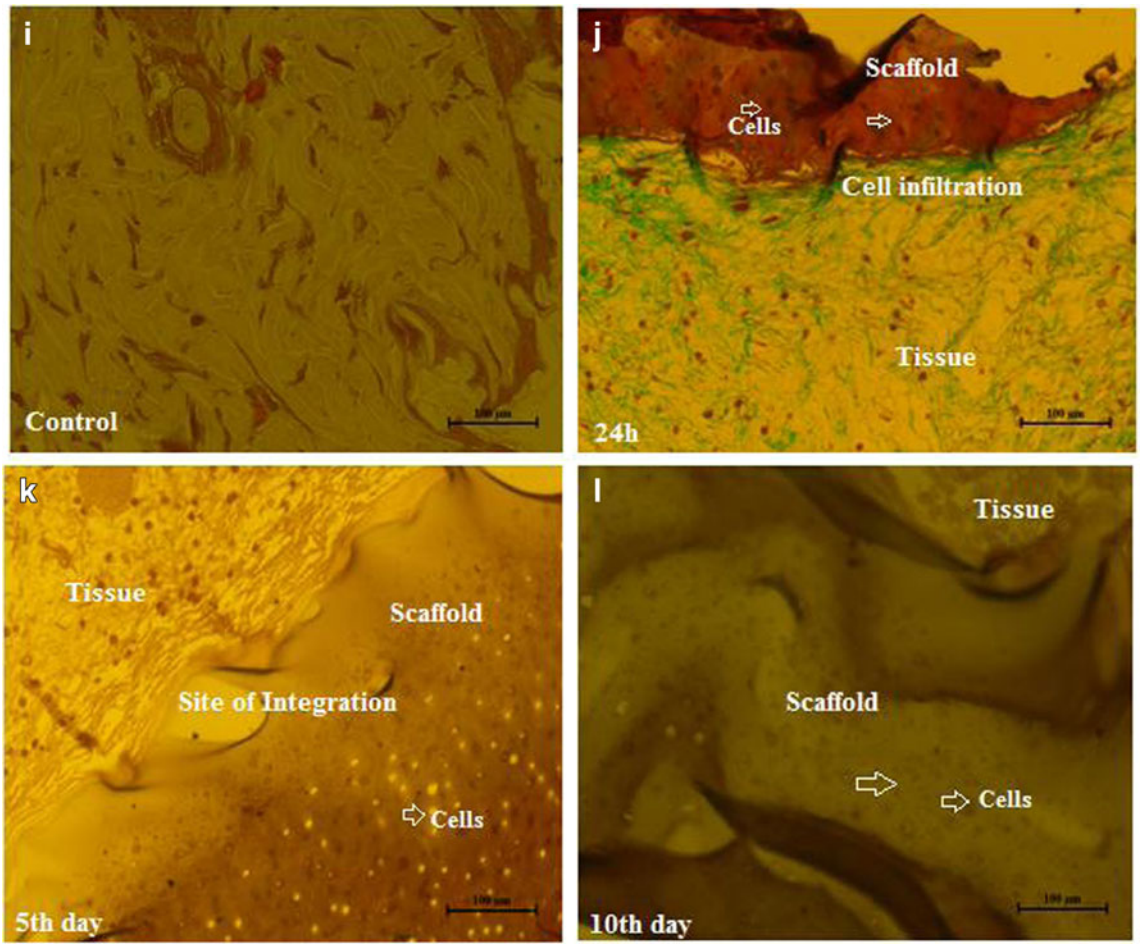

Fig. 10 (continued)

significantly higher at the first day of implantation in comparison to fifth and tenth days of implantation where insignificant amount of mast cells could be seen. In the case of HPMA-DMAEM hydrogel, connective tissue was found near the implanted material after the second week. The material was found to completely integrate into the host tissue at tenth day of implantation

Conclusion

In this study, we have fabricated dual-responsive hydrogel based on HPMA-co-DMAEM that exhibits drug release behavior that can be controlled by its pH sensitivity. The stability of the hydrogel changed with varying temperature, and the swelling of gels at low pH value can be attributed to controlled release profile. The synthesized hydrogels begin to swell below the physiological pH 7.42, and the maximum swelling was at pH 4–6, owing to tertiary amine groups on DMAEM. By modulating the ratio of DMAEM which play a role in the pH transition value of hydrogels, the hydrogels can be modulated, depending upon the desired application using the above phenomena of polymers. The biocompatible study indicated that the matrix can be easily utilized in the in vivo system without eliciting any adverse reaction to the surrounding tissue. The cell attachment, proliferation, and secretion of natural ECM were a clear indication of biocompatible nature of the synthesized matrix. The pH responsiveness with thermo-responsive behavior allows the matrix to become active with specific temperature or pH, giving a wider window for drug release. The drug delivery needs to mimic the biological property to some extent for various applications. The fabricated hydrogel could be used in cancer therapy as the DOX release profile, and the pH-responsive behavior of these gels can contribute to achieve precise targeting of cancer cells and tumor tissues without affecting the healthy surrounding tissues bared on difference in pH system. The stimuli-responsive HPMA/DMAEM scaffold with its biocompatible interconnected porous materials and mechanical stability could be an ideal scaffold for tissue engineering.

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