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An investigation study of gelatin release from semi-interpenetrating polymeric network hydrogel patch for excision wound healing on Wistar rat model

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ABSTRACT: Semi-interpenetrating polymeric network hydrogel patches are fabricated using poly(acrylamide) (PAm) and gelatin (G) in which poly(caprolactone) diacrylate is used as a crosslinker for PAm while gelatin is kept uncrosslinked. The healing efficiency of selected hydrogel dressing [$PAm_1G_1(0.5)$] is evaluated in comparison with control group (cotton gauze covered with 3M Tegaderm^{TM}). The sustained release of gelatin is found to extend from 4 to 15 days while maximum tensile strength stretched to 559 ± 12.5 kPa in $PAm_1G_{0.5}$ matrix, which reduced to 158 ± 6.1 kPa at higher gelatin content ($PAm_1G_{1.0}$). The higher wound contraction (34%), less inflammatory response, significant improvement ($P < 0.05$) in the collagen biosynthesis, and the granulation tissue formation are observed in PAm/G treated animals in comparison to control, as evidenced by quantitative enhancement of DNA (21%), hydroxyproline (28%), and hexosamine (41%). The histological examination of PAm/G hydrogel treated wound tissues shows enhanced re-epithelialization on day 8 and 12 post-wounding, in comparison to control group. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42120.

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INTRODUCTION

Wound repair is an essential process in maintaining tissue homeostasis in response to injury while wound management is a complex and challenging area of research, where selection of polymeric composition in the dressing as well as their physicochemical properties play a crucial role towards regeneration of nascent tissues.[1–4] Modern wound management assimilates certain essential qualities to the new generation dressing, includes, (i) mechanical integrity during handling in both dry and wet state, (ii) keep the aseptic surrounding in the injured zone, (iii) maintain a continuous hydrated and soothing environment around the injured tissue, and (iv) remain non-sticky and allow smooth peeling without injury to the newly formed tissues. With advancement of molecular understanding, it was found that cell interactive surface is useful for the attachment, proliferation, and migration of growing tissues/cells.[4–6] Among various type of dressings (viz. gauze, hydrogel, foam, ointment, sheet, films, hydrocolloid, etc.), hydrogels are found to be one of the most promising and versatile formulations for wound management. Their soft and elastic consistency resembles them to natural tissues and analogous to the extracellular matrix (ECM).[2,7–9] Additionally, water retention quality of hydrogels prevents tissue dehydration thereby, retains the essential components of blood and ECM, viz. enzymes, cytokines, and growth factors, into the wound bed. Consequently, moist dressing accelerates the rate of fibroplasia (fibroblast migration, proliferation, and secretion of ground substance) angiogenesis (neovascularization) and re-epithelialization.[1,4] However, researchers attempted various strategies to overcome limitations related to moisture balance, inflammation, infection, and healing promotion by means of antibiotics, silver compounds, anti-inflammatory agents, and also by designing of dressing with polymers possess water retaining capacity such as chitosan, polyacrylic acid, collagen, and alginate. Owing to bacterial resistance, delayed healing effect and toxicity aspects of antimicrobials, and mechanical constrains of polymeric devices, most of the systems having limited scope in modern wound management.[1,2,6,11]

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

Biocompatibility and biodegradability of the dressing offer advantage by providing cumulative spacing to the growing cells to migrate deep into the matrix and provide them a natural three-dimensional environment.[12] However, these biodegradable dressings are available in the form of films or sponges and are made up of biopolymers such as collagen, gelatin, alginate, hyaluronic acid, and chondroitin sulfate,[3,13–15] but such biopolymeric matrices have limited mechanical and physical integrity under aqueous/biological media.[8,9,16–18] Conversely, dressings from synthetic polymers (poly vinyl alcohol, poly acrylic acid, polyurethane, poly-lactic acid, poly-lactic-*co*-glycolide) have limited biological activity and need to modify with biomacromolecules for enhanced biological response.[15–20] With advancement of wound management, hybrid dressing has been evolved to overcome the lacunas of both the synthetic and natural polymers while retaining the promising features of the individual polymers.[9] Semi-interpenetrating networks are hybrid matrices made up of optimum combination of synthetic and natural polymers which have been designed to incorporate all promising properties in one system.[9,14,15] In our previous studies, some of hybrid hydrogel matrices have been synthesized with utilization of biocompatible polymers (approved by food and drug administration [FDA] of USA) which includes polyacrylic acid, 2-hydroxyl ethyl methacrylate (HEMA), polyacrylamide, and poly (caprolactone) diacrylate (PCL-DAr) along with gelatin as biopolymer to get tunable biological properties towards controlled drug delivery and tissue engineering applications.[7,9,11] Poly(acrylamide) hydrogel dressing has been showed remarkable ability for controlled drug delivery, cell proliferation potential for skin fibroblasts along with tunable biodegradability.[9,11]

In continuation of previous studies and incorporation of abovementioned properties towards designing of wound dressings, this research work explored the ability of polyacrylamide (PAm)/gelatin (G) hydrogel matrices as potential biomaterial for wound healing applications. Key parameters such as elasticity, availability of gelatin, moist environment, and biocompatibility around the wound bed have been addressed during studies. PAm chains were crosslinked with a hydrolytically degradable crosslinker, that is, PCL-DAr whereas peptide chains of macromolecule, that is, gelatin were intercalated in between PAm crosslinked network. Elasticity and gelatin release profile of these matrices were evaluated as a quality measure of dressing materials. The healing potential of selected hydrogel formulation [$PAm_1G_{1.0}$ (0.5)] has been evaluated on full-thickness excision wound. Wound contraction rate, histological parameters (epithelialization, granulation tissue formation, and inflammatory response), and biochemical measurements (collagen and DNA content, granulation tissue formation) were performed to investigate its potential in wound healing.

MATERIALS AND METHODS

Gelatin type A (From bovine skin, bloom strength ~300, IEP ~8.7) and acrylamide (crystals form; electrophoresis grade) were purchased from Spectrochem India, Mumbai, India. Tegaderm™ transparent non-adhesive waterproof film dressings were purchased from 3M™ Naxcare™. Acryloyl chloride and absolute ethanol were purchased from Merck, Darmstadt, Germany. Poly(caprolactone) diol (PCL-diol, M_w ~530 g/mol) were purchased from Sigma Chemical, St. Louis. Ammonium per sulfate (APS), tetramethylethylenediamine (TEMED), triethylamine (TEA), benzene, and chloroform were purchased from Qualizens India, Mumbai, India. All organic solvents were distilled before use.

Table I. Feed Composition of Semi-IPN Hydrogels[a]

Classification	Semi-IPNs code	Ratio (w/w) Acrylamide (Am)	Gelatin (G)	PCL-DAr Mol % of acrylamide
Group A	$PAm_1G_1(0.2)$	1	1	0.2
	$PAm_1G_1(0.5)$	1	1	0.5
	$PAm_1G_1(1.0)$	1	1	1.0
	$PAm_1G_1(2.0)$	1	1	2.0
	$PAm_1G_1(4.0)$	1	1	4.0
Group B	PAm_1G_0	1	0	0.5
	$PAm_1G_{0.1}$	1	0.1	
	$PAm_1G_{0.2}$	1	0.2	
	$PAm_1G_{0.5}$	1	0.5	
	$PAm_1G_{1.0}$	1	1.0	
	$PAm_1G_{2.0}$	1	2.0	

In addition, 0.2 mol % each of APS and TEMED was added to the feed composition to accelerate polymerization (quantity calculated with respect to Am concentration).

Synthesis of Crosslinker (PCL-DAr) and Semi-Interpenetrating Polymeric Network Hydrogel

PCL-diol was synthesized using PCL-diol (molecular weight ~530 g/mol) and acryloyl chloride by nucleophilic substitution reaction mechanism and characterized as per the method described in our previous studies.[9] Semi-interpenetrating polymeric networks (IPNs) hydrogel dressing of PAm/G was prepared by thermal initiated redox polymerization technique using PCL-DAr as a crosslinker while APS and TEMED as reaction accelerators. Aqueous gelatin (15% w/v) and PAm (50%) solution were prepared at 40°C with slow stirring in oxygen free water (N_2 bubbled) and gradually mixed with PCL-DAr solution (5% w/v solution in dimethyl acetamide) as per Table I. Free radical polymerization was initiated by adding APS and TEMED (0.2 mol % each) in polymer mixture. The petri plates/molds were kept in vacuum oven under N_2 atmosphere at 45°C for 6 h, for the formation of firm gels. Unreacted acrylamide and crosslinker was removed from the gels, their extensive washing with 30% (v/v) ethanol at room temperature and cut in suitable size by metal bore. Furthermore, sample specimens were dried in vacuum oven at 45°C for 24 h and kept in fused calcium chloride desiccators for further studies.

Tensile Strength

Tensile properties of hydrogel dressings were measured using uniaxial tensile tester (Tinius Olsen, Q4368, H5KS, Q-Mat 5.37 Software, PA). Test specimens were prepared by cutting the hydrogel sheet in its wet state, using a metallic bore in suitable shape (dumbbell) and sizes (according to ASTM-D638, 6.0 mm

thickness).[2] Experiments were performed using fully swollen samples at a constant humidity (75% RH) and temperature (25°C). Swollen specimens were first gripped in between two metallic jaws (upper movable and lower fixed one) of the mechanical tester and extended at a crosshead speed of 10 mm/min. To get a better grip and to avoid tearing of soft specimens by metallic grip, an additional tissue paper rolled over the gripping zone of specimens. To eliminate any chances of deformation errors, samples were stretched two times of their grip length. The tensile properties (tensile strength, max. elongation, and max. stress) were recorded by inbuilt software (Q-Mat 5.37).

Gelatin Release Profile

These semi-IPN hydrogel dressings keep the gelatin in its free uncrosslinked state. Its release profile was evaluated by immersing 6.0 mm diameter discs (approx. 10 mg) in 5.0 ml of freshly prepared PBS (pH 6.5), at 37 ± 0.2°C at 50 rpm in an incubator shaker. Initial gelatin content of the discs was calculated from their dry weight. 1.0 ml of aliquot was withdrawn at predetermined time intervals and replaced by equal volume of fresh media. Gelatin content was measured by Trinitrobenzene sulfonic acid (TNBS) assay method.[21] Briefly, to 1.0 ml of test specimen, 1.0 ml of 4.0% sodium bicarbonate (pH-8.5) and 1.0 ml of 0.5% TNBS solution (prepared from 5% stock solution) were added followed by incubation at 37 ± 0.2°C for 2 h with mild shaking. Afterwards, 1.0 ml of 10% sodium dodecyl sulfate solution and 0.5 ml of 1*N* HCl were added into above mixture with continued incubation at 60°C for next 2 h. Gelatin was quantified by measurement of optical density at 336 nm using UV–visible spectrophotometer. A standard curve of known concentration of gelatin (10, 20. 30, 40, and 50 μg/ml) was made under same processing conditions of TNBS assay. The dynamics of release pattern of gelatin from the various crosslinked networks was studied using the following equation:

$$f = kt^n. \quad (1)$$

"f" is the fractional solute release (M_t/M_∞) where, M_t is the fraction of gelatin released at time t, M_∞ is the initial concentration of the gelatin in the hydrogel disc, n is a kinetic constant characteristic of the solute/polymer system, n is an exponent which characterize the mechanism of gelatin release (i.e., either diffusion control or both diffusion and erosion control).[22,23]

In Vivo Wound Healing Experimentation

Experimental Protocol. In this study, healing potential of hydrogel based polyacrylamide/gelatin dressing was evaluated in full-thickness excision wounds using male Wistar rats of 200 ± 20 g weight. All the experiments were performed according to guidelines of animal ethical committee (06/362/IAEC) of All India Institute of Medical Sciences (AIIMS), New Delhi, India. Animals were kept in separate cages under controlled environment conditions (25°C and suitable humidity). Animals were divided in two groups; (1) control (moist cotton gauze; moisture in the wound bed was maintained by covering it with 3M™ Tegaderm™ membrane), (2) hydrogel dressing (PAm/G); covered with 3M™ Tegaderm™ membrane; in each group 12 rats were taken. Each group was divided in two sub-groups; group-A (six rats) for wound contraction and histopathological evaluations and group-B (six rats) for biochemical evaluation of healing biomarkers.

Excision Wound Model. The animals were anesthetized by an intramuscular injection of 100 μl of 0.5% (w/v) Ketamine solution (HypoKet®, Chandra Bhavan Pharma, Mumbai, India). Hairs from the dorsal side of the rat were shaved with electric hair clipper (Moeser, Unterkirnach, Germany) and the underlying skin was cleaned with 70% ethanol. A full-thickness circular wound of 2.0 cm diameter was created using sterile surgical scalpel blade and scissor up to the depth of subcutaneous tissues. After wound creation, dressing was applied to the control (cotton gauze) and treated (PAm/G hydrogel dressing) groups [Figure 1]. Sterilized cotton gauze was soaked in saline in control group whereas in PAm/G hydrogel dressing was applied in their equilibrium swelling state and both groups were covered with transparent Tegaderm™ film to keep moist environment beneath the dressing. Dressing was further covered with microporous adhesive paper to keep it in its position up to next changing period.

Evaluation of Pro-Healing Parameters. ***Measurement of percentage wound contraction.*** Dressings were changed on day 3 and 8 post-wounding and kept on the wound surface till 12th day. The progression of wound contraction was measured by marking the wound edges of healed boundary by a marker on a transparent tracer paper and measured planimetrically.[2,24,25] The % wound contraction was calculated using the formula:

$$\%\text{Wound contraction} = \left(\frac{A_i - A_t}{A_i}\right) \times 100, \quad (2)$$

where A_i is the initial wound area at day zero and A_t is the area of wound on day when dressings were changed (days 3 and 8 post-wounding).

Biochemical and histological examinations. A group of animal was sacrificed on eight day post-wounding and the regenerated wound tissue samples were collected and assessed for pro-healing biochemical parameters. The hydroxyproline (HP), hexosamine (HA), and DNA contents were determined as per established procedures.[25–30]

For histopathological evaluation, animals were sacrificed on days 3, 8, and 12. The biopsies of the peripheral healed tissues with central injured area were performed and were fixed with 10% phosphate-buffered formalin solution. These samples were embedded in paraffin and sectioned (4.0 μm) using microtome (RM2235, Leica, Germany).The hematoxylin and eosin (H&E) stained sections were examined under light microscope independently by two individuals for semi-quantitative grading of different parameters for assessment of healing process (acute/chronic inflammation, fibrosis, number of fibroblast cells, neovasculature, and collagen fibrils).[2,20]

Morphometric Measurements and Analysis. All morphometric parameters were measured with Image Analyzer (Olympus Microscope BX61, Japan) using image analyzing computer program (Image-Pro Plus 6.3 NIH, Public domain). All histological sections were assessed through the center of the wounds to obtain maximum wound diameter. The measurements were

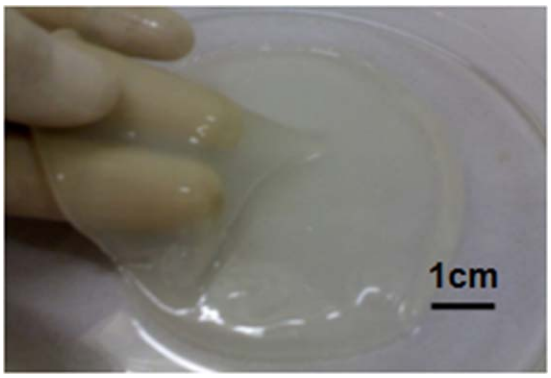

Figure 1. PAm/G semi-IPN hydrogels in equilibrium swelling state. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

taken three times, by examining the slides in random sequence, blinded to treatment. The thickness of the newly formed epidermis was measured at 1.0 mm interval, and the mean was calculated. The density of the granulation was evaluated by taking average number of cells in six high power fields (60× objectives), midway in the wound bed. We also counted the number of vascular spaces in six high power fields (60× objectives), midway in the wound bed. Dermal thickness was determined at the center of each section, vertically, from the surface of granulation tissue to the margin of dermis and subcutis. Eschar where present, was not included in this measurement.[20,30,31]

Statistical Analysis

Data are expressed as mean ± SE, and statistical significance between experimental and control values was analyzed by one-way ANOVA followed by Dunnett's test using GraphPad Prism 2.01 (Graph Pad Software, La Jolla, CA). A *P*-value 0.05 was considered statistically significant.

RESULTS

Gelatin Release Kinetics

Being a natural peptide sequence (RGD), gelatin is a very useful biopolymer towards cell proliferation thereby for progression of healing.[7,14] To get its continuous availability in the wound bed, varying concentration of crosslinker and its ratio with PAm were tested for weight loss/gelatin release profile.

The effect of crosslinker concentration and PAm/G ratio on gelatin release profile from various formulations was depicted in Figures 2(I) and 3(I). Increasing crosslinker concentrations from 0.2 to 4.0 mol % (at constant PAm/G ratio of 1 : 1) strengthen the crosslinking network, which resulted into increased stability of PAm/G matrices up to 8th day.[9] As a result, the gelatin release decreased from nearly 68% to 37% on day 2, from 76% to 14%, on day 6 and from 100% to 17% on day 10. Conversely, with increasing crosslinker content from 2.0 and 4.0 mol % [i.e., $PAm_1G_1(2.0)$ and $PAm_1G_1(4.0)$, respectively], gelatin release reduced from ~39% to less than 30%, on day 10. Under second set of experiments, when G/PAm ratio decreased from 1/1 to 0.1/1 [from PAm_1G_1 to $PAm_1G_{0.1}$], only ~21% gelatin was found to release after 10 days [$PAm_1G_{0.1}(0.5)$].

At low crosslinker concentration (0.2 mol %), a loosely crosslinked network was formed which comprised of larger pore size. This resulted as increased swelling of matrix in compare to those which contain higher crosslinker content (4.0 mol %). Conversely, decreased swelling and formation of a dense network with decreasing gelatin content resulted into decreased diffusivity of the buffer medium into the PAm network. In other words, decreasing G/PAm ratio reduces the distance between two PAm chain and get tougher for gelatin to come out of PAm network. The assessment of gelatin release kinetics from degradable matrices was performed as per the eq. (3):

$$\ln f = \ln K + n \ln t. \quad (3)$$

K is Korsmeyer release constant dependent on the properties of solute and polymer. The n is an exponent which defines the mechanism of gelatin release whether it is diffusion controlled (chain relaxation) or both diffusion and erosion controlled.[7,22,23] When $n < 0.5$, Fickian diffusion suggested while in case of $0.5 < n < 1.0$, non-Fickian mechanism is assumed. The value of (n) was observed from the slop of the plot of logarithm of release rate (f) and logarithm of time (t). The diffusion exponent (n) and regression coefficient (r^2) value are tabulated as Table II. The results showed that from $PAm_1G_1(0.5)$ and $PAm_1G_1(1.0)$ hydrogels, gelatin was released with non-Fickian

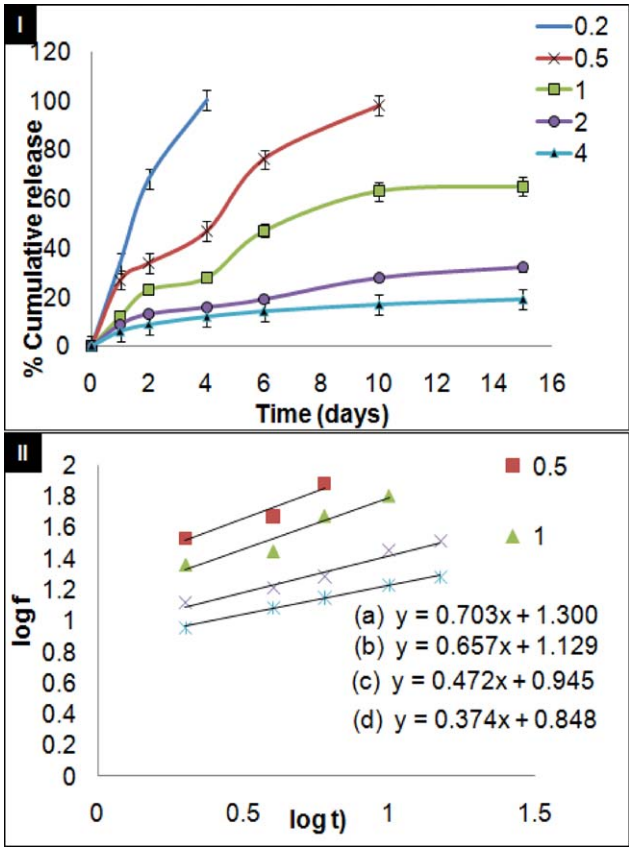

Figure 2. (I) Gelatin release profile and **(II)** release kinetics of gelatin (log % CR vs. log t) from biodegradable hydrogel network of PAm/G with respect to crosslinker concentration from 0.5 to 4.0 mol % of Am in PBS (pH 6.5), 37 ± 0.2°C (at constant PAm/G ratio [1 : 1]). Inset indicates the crosslinker concentration (data were used as mean ±SD, $n = 3$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table II. Gelatin Release Profile from PAm/G Semi-IPNs with Respect to Feed Ratio; Diffusion Coeficient (n), Regression Coefficient (r^2), and Release Mechanism of Gelatin

Formulation	Diffusion exponent value (n)	Regression coefficient (r^2)	Release mechanism
PAm_1G_1 (0.5)	0.703	0.913	Diffusion and erosion
PAm_1G_1 (1.0)	0.657	0.972	Diffusion and erosion
PAm_1G_1 (2.0)	0.472	0.987	Diffusion controlled
PAm_1G_1 (4.0)	0.374	0.986	Diffusion controlled
$PAm_1G_{0.5}$ (0.5)	0.546	0.981	Diffusion and erosion
$PAm_1G_{0.2}$ (0.5)	0.404	0.976	Diffusion controlled
$PAm_1G_{0.1}$ (0.5)	0328	0.992	Diffusion controlled

anomalous mechanism ($n > 0.5$) where diffusion and surface erosion mechanisms work together for its release [Figure 2(II)]. Same mechanism was also noticed from $PAm_1G_{0.5}$ hydrogels [Figure 3(II)]. Conversely, from PAm_1G_1(2.0) and PAm_1G_1(4.0) hydrogel network, gelatin was released with Fickian diffusion pattern ($n < 0.5$) [Figure 2(II)], and same pattern was followed by $PAm_1G_{0.1}$ and $PAm_1G_{0.2}$ [Figure 3(II)] hydrogels network. The dense crosslinked network of these matrices did not allow them to degrade in the extent as found in case of matrices with low crosslinker concentration (0.2, 0.5, and 1.0 mol %). Therefore, in these matrices gelatin release was controlled by relaxation of polymeric network only. PAm_1G_1(0.2) matrices released more than 60% of its entrapped gelatin in two sampling (24 h and 48 h) and two point linear equation cannot make it rationale to depict release mechanism. Therefore, its gelatin release data was not been interpreted in the Table II and in Figure 2(II).

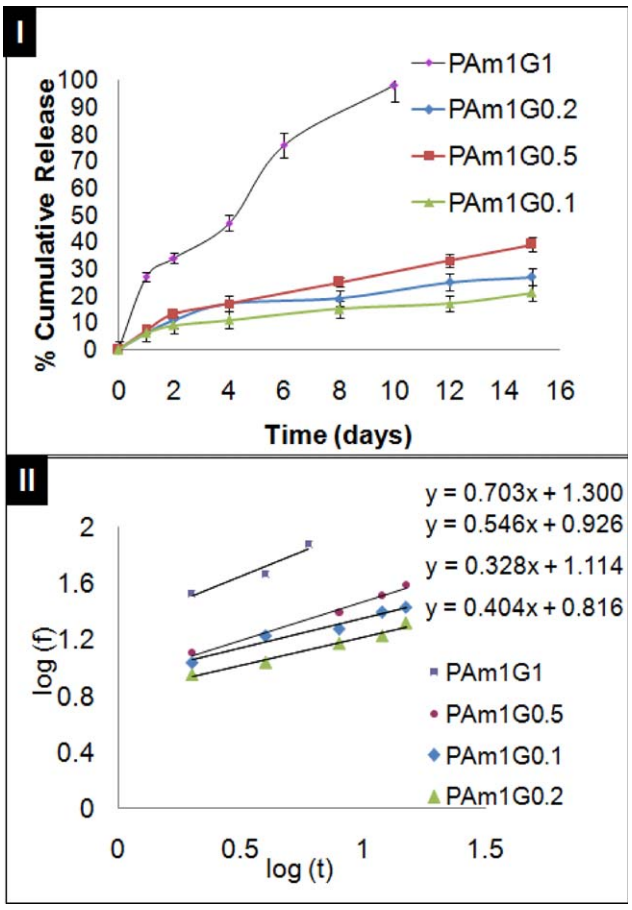

Figure 3. (I) Gelatin release profile and (II) release kinetics from biodegradable semi-IPN hydrogels of PAm/G with varying gelatin concentration PBS (pH 6.5), 37°C while Am and crosslinker content (0.5 mol % of Am) were constant (a) PAm_1G_1, (b) $PAm_1G_{0.5}$, (c) $PAm_1G_{0.2}$, (d) $PAm_1G_{0.1}$ (data were used as mean ±SD, $n = 3$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Tensile Properties

Mechanical strength is major limiting issue for hydrogel formulations in support of their biomedical applications in particular, as a dressing. Therefore, this research work exclusively addressed this parameter with varying formulation parameters.

At a constant crosslinker concentration (0.5 mol %), the effect of gelatin content on tensile properties of PAm/G semi-IPN dressing was studied using uniaxial tensile testing machine (Figure 4 and Table III). Notably, with increasing G/PAm ratio

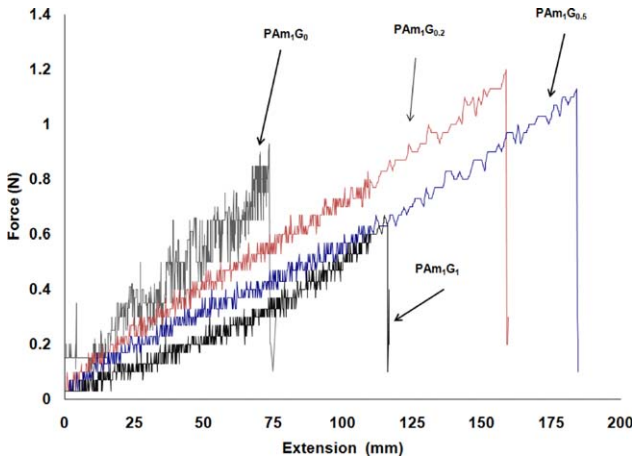

Figure 4. Effect of gelatin content (from 0 to 1.0 w/w ratio with PAm) on elasticity of PAm/G semi-IPN hydrogels (crosslinker concentration was constant, i.e., 0.5 mol %). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

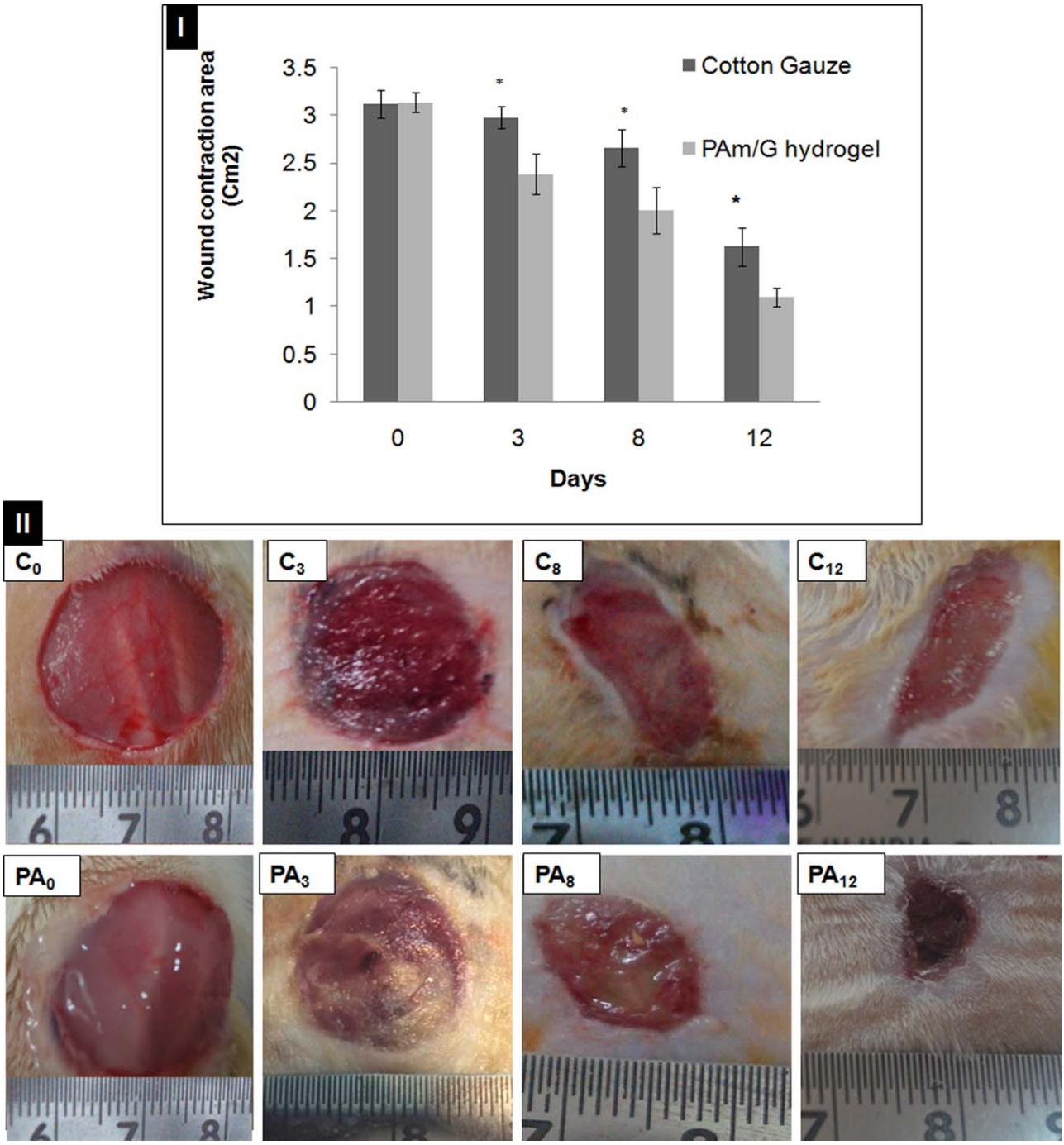

Figure 5. **(I)** Photographic representation of wound contraction on control group (C_0, C_3, C_8, C_{12}) and PAm/G semi-IPN hydrogel dressing PAm_1G_1 (0.5) (PA_0, PA_3, PA_8, and PA_{12}) on days 0, 3, 8, and 12, respectively; **(II)** comparative graphical representation of wound contraction area after application of cotton gauze and PAm/G semi-IPN hydrogel dressing (*$P < 0.05$ in comparison to cotton gauze); subscription denote the day of dressing application). Data were collected as mean ±SD; $n = 6$ rats. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(from 0 : 1 to 0.5 : 1), maximum elongation, tensile strength, and maximum stress values increased from ~70% to ~244%, ~194 kPa to ~559 kPa, and 0.32 N to 1.24 N, respectively. Further increment in G/PAm ratio, that is, 1 : 1, leads to steep declined of these properties from ~244 to ~132%, ~559 kPa to ~158 kPa, and from ~1.24 N to ~0.43 N, respectively (Table III). Noticeable that inclusions of gelatin chains not only improve the elasticity but also strengthen the PAm crosslinked network by means of physical interaction (hydrogen bonding).[9] In addition, beyond certain concentration of gelatin, tensile strength of the matrices decreases, as indicated in Figure 4. This may be due to stronger ionic repulsion of gelatin functional groups (—COOH, —NH_2) which overcome the physical crosslinking, led to diminished tensile properties.

Table III. The Effect of the Gelatin Content on Tensile Properties of PAm/G Semi-IPN Hydrogels

	Tensile strength (kPa)	Maximum elongation (%)	Max. force (N)
PAm_1G_0 (0.5)	194.5 ± 7.8	70 ± 3.7	0.32 ± 0.05
$PAm_1G_{0.2}$ (0.5)	432.8 ± 8.2	266 ± 9.6	1.14 ± 0.06
$PAm_1G_{0.5}$ (0.5)	559 ± 12.5	244 ± 11.4	1.24 ± 0.18
$PAm_1G_{1.0}$ (0.5)	158 ± 6.1	132 ± 6.2	0.43 ± 0.1

Data were used as mean ±SD, $n = 5$.

In Vivo Efficacy of Semi-IPN PAm/G Hydrogel Dressing

However, from tensile studies, hydrogel matrices with 1 : 1 PAm/G ratio showed less tensile strength in comparison to matrix with 1 : 0.5 PAm/G ratio, but from *in vitro* degradation studies,[9] it was observed that matrices with 1 : 1 PAm/G (with 0.5 mol % crosslinker concentration) degraded within 4 days which released its gelatin content during that period (Figures 2 and 3). Therefore, with consideration of degradation profile and gelatin release profile, PAm_1G_1 hydrogel matrix with 0.5 mol % crosslinker concentration was selected as an experimental dressing for excision wound healing.

Wound Contraction. As a result of better healing environment (moist, protective, biocompatible, and ECM mimetic) around the growing tissues, significantly ($P < 0.05$) improved healing was observed in animal groups treated with PAm/G dressing as depicted in Figure 3(I,II). PAm/G hydrogel dressing showed nearly 30% enhancement in wound contraction area (~65% healing) (area remain unhealed was 1.1 cm^2) in comparison to cotton gauze which showed 1.56 cm^2 unhealed area on day 12 post-wounding (Table IV).

Biochemical and Histological Examination. Post-operative biochemical examination of tissue includes assessment of DNA, HP, and HA content. A significant enhancement in DNA (21%), HP (41%), and HA content (24%) was found in animal groups treated with PAm/G hydrogel dressing, in comparison to control group (Table IV). Improvement in DNA content represents enhancement in the mitotic activities of the growing cells in the wound bed. Improvement in HA content represents that granulation tissues were formed in a higher extent during the healing period, which includes ECM, fibrous tissues, and glycosaminoglycans. Hydroxyproline represents the collagen content in the regenerated wound tissue.

Figure 6 showed histological studies of wound tissues on days 3, 8, and 12 after application of control and hydrogel dressings at low power (4×) [6(I)] and high power (20×) [6(II)] magnifications. In Figure 6(I), low power photograph (4×) of the superficial zone of the wound bed showed pronounced polymorphonuclear leukocytes (PMN) infiltration on top and necrotic tissue beneath [Figure 6(I) (C-ii, iii)]. From high power field (20×) observations of H&E stained section [Figure 6(II)] of control group, necrosis and PMN infiltration on surface and vascular scar tissue in the wound bed were noticed while deeper zone of the wound bed was found with numerous dilated blood vessels (BV), epithelialization (EP), fibroblast proliferation, and collagen formation on day 12 (also mentioned in Supporting Information Figure S1). PAm/G hydrogel dressing showed enhanced healing with faster re-epithelialization, less inflammation, higher granulation tissue formation, and reduction in collagen deposition on day-12 post-wounding. The scoring of histopathological examination as (−), (+), (++), and (+++) was mentioned in Table V.

A minimum of 50 high power fields (hpf; 40×) were assessed for the grading. Acute inflammation was graded by average number of acute inflammatory cells (neutrophil)/hpf; less than 1/hpf (grade ±), 1–2/hpf (grade +), 3–4/hpf (grade ++), 5–6/hpf (grade +++), and >6/hpf (grade ++++). Similar grading was followed for chronic inflammation by assessing the number of chronic inflammatory cells (lymphocytes, histocytes, and plasma cells)/hpf. The degree of edema was assessed by noting the intercellular space in the upper dermis. Edema was graded as ± if the loose space was seen in <5 out of 50 hpf, + in 5–10 hpf, ++ in 11–20 hpf, +++ in >20 hpf. Granulation tissue was graded as + with <25 fibroblasts with large vesicular nuclei cells/hpf, ++ 26–50 cells/hpf and +++ with

Table IV. Comparative Analysis of the Effect of PAm/G Semi-IPN Hydrogel Dressing Wound Contraction Area on Days 0, 3, 8, and 12 and, on DNA, Hexosamine (HA), and Hydroxyproline (HP) Content on Day 12; (*$P < 0.05$) in Comparison to Control Group; Data Were Used as Mean ±SD, $n = 6$ Rats

	Wound contraction (cm^2) (wound area remaining to heal after specific time point)				Biochemical analysis		
					DNA (mg/g of dry tissue)	HA	HP
Days	0	3	8	12	On day 12		
Control	3.14 ± 1.1	2.98 ± 2.1	2.66 ± 2.4	1.56 ± 0.17	2.49	0.669	23.33
Hydrogel dressing PAm_1G_1 (0.5)	3.14 ± 0.14	2.39 ± 0.11	2.01 ± 0.19	1.1 ± 0.2*	3.01*	0.835*	33.7*

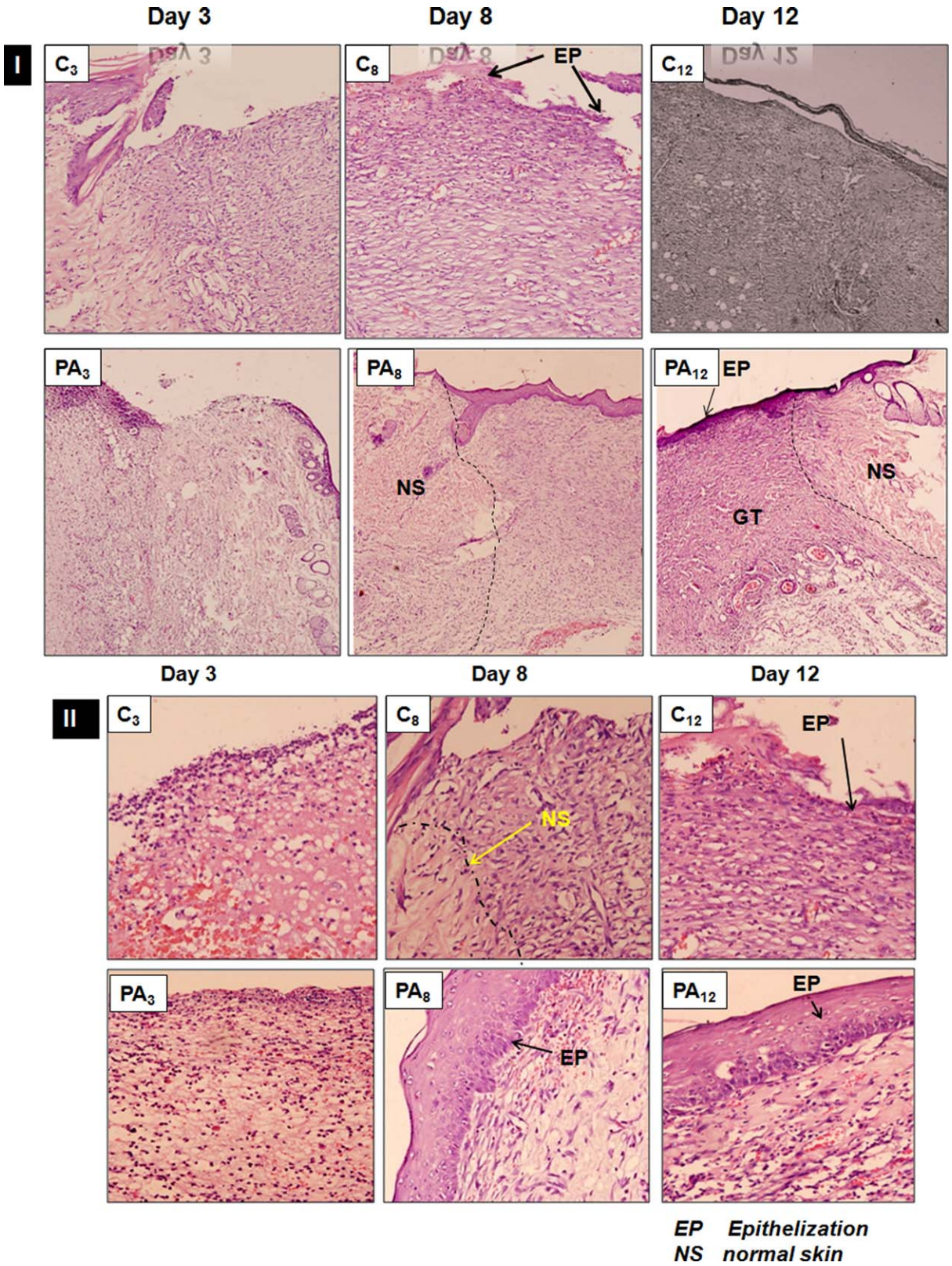

Figure 6. H&E stained photomicrographs of wound tissues in control group (C) and PAm/G hydrogel dressing group (PA) on day 3, 8 and 12 at (I) 4× and (II) 20× magnifications. Advanced and phenomenal epithelialization as well as comparatively lesser inflammatory response was observed in PAm/G semi-IPN hydrogel dressing (PA_8 and PA_{12}) in comparison to control group (C_8 and C_{12}). Control group also showed comparatively higher inflammatory response on days 3 and 8 (C_3 and C_8). (GT—granulation tissue, EP—epithelialization, NS—normal skin). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table V. Graded Response of Prohealing Parameters in Cotton Gauze (Control), PAm/G Semi-IPN Hydrogel Dressing

	Day 3		Day 8		Day 12	
	Control	PAm/G Semi-IPN	Control	PAm/G Semi-IPN	Control	PAm/G Semi-IPN
Acute inflammation	+++	++	++	±	±	–
Chronic inflammation	+	±	+	±	+	-
Edema	++	+	+	-	±	±
Granulation tissue formation	+	++	++	++	+	++
Collagen deposition	+	+	++	++	+	++
Capillary formation (angiogenesis)	+	++	++	+++	++	+
Epithelialization	±	++	+	+++	++	+++

(+++) referred as maximum response while (–) inferred for minimum response.

>50 cells/hpf. If extracellular collagen deposition was seen in <25 out of 50 hpf then the collagen deposition was graded as + and if in 25 or more hpf then graded as ++. The angiogenesis was assessed as the number of capillaries in upper dermis. It was graded as <5/hpf (±), 5–10/hpf (+), and >10/hpf (++). The re-epithelialization was evaluated by assessing the regenerating the epidermal layers. It was graded as ± if less than two cell layer, + with two to three cell layer, ++ with three to four cell layer, and +++ with five or more cell layer.[2]

It was suggested that wound closely resembles to the surrounding normal dermal architecture. Reduced granulation tissue and collagen deposition in the later stage of healing were an indication of maturation of wound tissue.[2,31,32] Decreasing number of neovasculature on day 12 in hydrogel dressing treated groups is a healing biomarker as maturation of blood vessels. Parallel orientation of collagen fibers with epithelium and their compact deposition indicates the improvement of quality of healing (Supporting Information Figure S1).

With maturation these immature plump fibroblasts became mature fibroblast with spindle shaped nuclei and eosinophilic abundant cytoplasm which represented collagenization. With extracellular collagen deposition the number of mature fibroblast decreased/hpf with increase in intercellular eosinophilic hyaline material.

DISCUSSION

Because of huge demand and higher expectation form the patients; development of new bioactive wound dressings is a very growing area of research. The current research work designed an ideal wound dressing is that of a biocompatible and biodegradable scaffold through which skin cells will interact, migrate, and populate in a natural manner. In addition, we addressed the effect of polymer composition on physicochemical and biological properties of the dressing. The new generation dressing must imbibe prerequisites of a bioengineering product as well as it fulfill all essential qualities such as aseptic, moist, and soothing environment around the injured tissue throughout the application. Additionally, dressing must peel out softly without injury to the newly formed tissues and also support the growing tissues towards healing. Furthermore, dressing should be capable of delivering therapeutic substances in a control manner.[2,33] We explored biocompatible biomaterials for the development of a dressing to contain most of the required features of an ideal dressing. The selection of polymers was geared with consideration of desirable requisites of dressing matrices like, biocompatibility, biodegradation, tensile properties, water absorption capacity, and availability of bioproteins for cell attachment and exhilaration of healing process, the end application. Inherent water retention property of PAm/G hydrogel matrices offered as a reservoir for the vital ECM components (glycosaminoglycans, growth factors, and cytokines) in the wound bed which are crucial for the acceleration of fibroplasia (fibroblast migration, proliferation, and secretion of ground substance), neoangiogenesis (neovascularization), and re-epithelialization.[1,2,32] Angiogenesis is a very crucial factor in wound healing progression as it ensures the availability of oxygen and other vital nutrients for the newly forming granulation tissues.[34] Kapoor *et al.*[31] have reported that new blood vessel formation in wound bed begins within 3 days, peaks at day 7, and thereafter resolves, resulting in the characteristic avascular scar. Histological studies of biopsied wound tissue of PAm/G dressing treated group showed only few blood vessel on day 12 (Supporting Information Figure S1 and Table V), which indicated that optimum healing environment was offered by these matrices.

Tensile properties of PAm hydrogel were enhanced by incorporation of gelatin. Gelling properties and participation in intermolecular and intra molecular H-bonding with free functional groups of PAm network are two main reasons for improved elasticity of PAm/G network. However, above a certain ratio of PAm and gelatin, tensile behavior changed in reverse direction. Therefore, an optimized PAm/G ratio (1 : 1) with 0.5 mol % crosslinker concentration was selected to study their healing efficacy. Attributed to biodegradable property and porous-scaffold like architecture,[9,11,35] hydrogel dressing acts like a template and provides cumulative spacing to the nascent tissues[32,35] (myofibroblasts and neovasculature) for three-dimensional migration thereby, accelerates in-growth and proliferation of cells from surroundings. Gelatin, like other ECM protein (i.e., collagen and fibronectin), regulates tissue regeneration process by adsorbing onto the cell surface receptors (integrins) and triggers the cell signaling cascades for further triggering of gene expressions by adsorbing onto the cell surface receptors

(integrins).[7–12,35–38] Though gelatin is not a native protein, but it carries RGD peptide sequence and provides active sites for the newly generated tissues. These unique features are very much appreciative during wound healing for cells attachment and proliferation. Moreover, during biodegradation process, gelatin was released from the hydrogel into the wound bed in a time-dependent manner thereby, continuously available for the growing tissues. As a result, a faster and natural healing took place. After carrying the migrated cells from wound surrounding, dressing degraded and become a part of granulation tissue which was supposed to help in regulation of collagen remodeling.[3,11]

CONCLUSIONS

Tissue compatible, elastic, and biodegradable hydrogel based dressing of PAm/G was found very effective in full-thickness excision wound healing by providing adequate moist environment as well as nutrition to the growing tissues. Covering of dressing with Tegaderm™ prevents the excessive loss of moisture and this strategy allows the wound to keep in hydrated state for desired time period. Tensile strength, elasticity, and gelatin release rate were observed to be regulated by crosslinker concentration and PAm/G ratio. Inclusion of all desired properties in a single dressing makes it an ideal formulation for excision wound management.

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