In Vitro and in Vivo Studies of pH-Sensitive GHK-Cu-Incorporated Polyaspartic and Polyacrylic Acid Superabsorbent Polymer

Shilpa Sharma,‡†‡∥§ Mohammad Faiyaz Anwar,‡ Amit Dinda,‡∥ Maneesh Singhal,§ and Amita Malik†‡§

‡Department of Chemistry, Dyal Singh College, University of Delhi, New Delhi 110001, India
§Department of Pathology and ¶Department of Plastic and Reconstructive Surgery, All India Institute of Medical Sciences, New Delhi 110029, India

Supporting Information

ABSTRACT: The main aim of this study was to evaluate the in vitro and in vivo efficiency of the polyaspartic acid- and acrylic acid-based superabsorbent polymer. The synthesized polymer was first investigated to check the blood compatibility by protein adsorption and blood clotting tests. Further, the GHK-Cu peptide was incorporated within the polymer and release studies were performed to evaluate the drug-delivery efficiency of the superabsorbent polymer. The polymer with best peptide release results were further used for in vivo analysis for wound healing. The healing efficiency of polymer with and without peptide was analyzed using wound closure, biochemical assay, histopathological, and toxicity studies.

1. INTRODUCTION

Human body has strong immune system with self-healing ability. The skin protects the body from the external environment.1 Wound healing is an active process that comes in action once the protective barrier is broken.2 Severe burns and associated wounds can cause pathological stress response and also large scars influencing patient’s life.3 Different levels of burns require different care and attention; for example, firstdegree burns, also known as superficial burns, cause minimum damage.4,5 They are usually treated with homemade remedies and require less healing time. Secondary and third-degree burns are severe and carry the most risk for compilation such as infection and blood loss, resulting in chronic wounds.6,7 Wound management has gained importance in recent years. The healing techniques have improved over time. Modern healing techniques overshadow the traditional methods. In the early 1980s, the traditional wound care products involved like gauze, cotton, and sponge, which were replaced with advanced wound care products like bioactive materials after 2000.8 These materials possess an active component which delivers during wound healing. The main property of bioactive material is their ability to combat bacterial infections, which promotes wound healing. These severe burn wounds are treated with different biomaterials like hydrogels, superabsorbent polymer (SAP), scaffolds, and skin grafting.9–11

Superabsorbent polymers are three-dimensional cross-linked polymeric chains with high fluid absorption capacity.12 These special absorbing materials are used in a variety of applications ranging from personal hygiene products and agriculture to tissue engineering and other biomedical products.13 Due to these applications, these polymers are now being used in the medical field, including wound healing. Global SAP market size was estimated at 2.07 million tons in 2014 and is projected to exceed 3.1 million by 2023.

The majority of synthesized SAPs are based on petroleum-based monomers like acrylic and acrylamide because of good mechanical strength and absorption property.14 The drawback of using this kind of monomer is that they lack biocompatibility and biodegradability. Thus, scientists today have changed their focus from synthetic SAPs to bio-based SAPs synthesized from starch, cellulose, natural gums, chitin, chitosan, alginites, and polyelectrolytes.15–18 Bio-based SAP provides environmentally sustainable alternatives to fossil-based material. These biopolymers are potent applicants for synthesizing biocompatible polymers but lack mechanical and absorption properties. Natural polymers are like poly(amino acids), polysaccharides, and protein have been known to exhibit biochemical and biological properties.19,20 Poly(amino acids) can serve as an alternate to petroleum-derived polymers because of their biocompatibility and biodegradability. Different poly(amino acids), namely, polysaccharic acid (PASP), polyglutamic acid, and poly(lysine) are widely being studied, resulting in bioactive polypeptides.20–22 These polypeptides...
have biofunctionality, which separates them from other synthetic polymers, and because of this, they are now being used in tissue engineering, regenerative medicine, drug delivery, gene delivery, and wound healing.22–24 Different synthetic and natural materials are being used for SAP synthesis. Today research is more focused on hybrid polymers where natural and synthetic polymers cross-link to form hydrogels with high absorption and good mechanical properties. Furthermore, the potential applications of these hybrid polymers in medical, healthcare, and pharmaceuticals are also expected to benefit the growth of SAP market.25,26

SAPs are also referred to as stimuli-responsive polymer as they respond to change in external stimuli like pH, temperature, moisture, and magnetic or electric field. These polymers produce changes on micro- or nanoscale like change in morphology, molecular bonding, and molecular motion.27,28 Recently, stimuli-responsive polymers have attracted great interest because of in vivo applications. These polymeric gels are being used to store, protect, and release drug at the target site.29 Not only gels but also peptides, metalloprotein carriers, and cross-linked self-assembled peptide-based materials are being used as drug carriers.

Wound healing is a complex process that involves sequential deposition of extracellular matrix deposition like collagen and fibronectin. This deposition of matrix molecules helps in the formation of new tissue. GHK is a tripeptide complex that has been used in the cosmetic industry since 1973. GHK complex exhibits high affinity for copper(II) ions forming GHK-Cu.33 This metal–peptide complex promotes tissue regeneration and acts as copper delivery agent required for cellular functions. Use of GHK-Cu instead of GHK has more potent application as this copper peptide complex elevates copper deficiency with no oxidative damage.34 This copper tripeptide complex in wound healing plays an important role as it stimulates the production of collagen and helps in resuming the blood flow into damaged tissue. It also aids in improving the expression of basic fibroblast growth factor and vascular endothelial growth, which forms blood vessel.35

In our previous work, we discussed the synthesis of the superabsorbent polymer using polyaspartic acid as natural polymer and acrylic acid (AA) as synthetic monomer, which were cross-linked with ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane triacrylate (TMPTA) to form superabsorbent polymer. The swelling characterization of the polymer was done by exposing them to different physiological fluids like water, saline, glucose solution, and in solutions of different pH values. The synthesized polymers were evaluated by physiochemical techniques like Fourier transform infrared (FTIR), thermogravimetric analysis, and scanning electron microscopy.36

However, our present study is an extension to our previous work, which explained the in vitro and in vivo study of superabsorbent polymer synthesized from polyaspartic acid and acrylic acid. Our current study focuses on encapsulation and release studies of GHK-Cu peptide. GHK-Cu peptide has been used for wound healing (in vivo studies) and has produced satisfactory results.37 In our research work, we focus on using GHK-Cu as drug moiety and will evaluate the peptide release on burn wounds. The polymer with and without peptide was then further evaluated for in vivo studies. The in vivo study includes wound closure, biochemical assay, histopathological examination, and toxicity studies.

2. RESULTS AND DISCUSSION

2.1. In Vitro Blood Compatibility of the Polymer. 2.1.1. Blood Clotting Test and Bovine Serum Albumin (BSA) Adsorption/Protein Adsorption. The blood compatibility of the polymeric material has been evaluated by studying the blood clotting and protein adsorption test. The blood clotting analysis explains the antithrombic property of the polymer, whereas the bovine serum adsorption helps in explaining thrombosis. Both these studies help in choosing the polymer to be used further for in vitro and in vivo studies.

The bar graph in Figure 1 clearly shows the blood clotting index and protein adsorption values of the superabsorbent polymers synthesized by using two different cross-linkers EGDMA and TMPTA.

The superabsorbent polymers were synthesized with polyaspartic acid and neutralized acrylic acid using different cross-linkers and initiator. The cross-linkers used for this study were ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane triacrylate (TMPTA), a difunctional and trifunctional, respectively. The main objective of synthesizing superabsorbent polymer was to use a polymer with moisture-retaining capacity. As moisture accelerates wound healing, the polymers used for wound healing should be designed in such a manner that it has good absorption and high biocompatibility. The biocompatibility is explained by BCI and protein absorption value in terms of thrombogenicity. Biocompatibility studies were performed to evaluate the biocompatibility properties of polyaspartic acid- and acrylic acid-based polymer with EGDMA and TMPTA cross-linkers. The synthesized

Figure 1. (a) Blood clotting index and (b) protein adsorption of polymer samples.
polymer should have a high BCI (%) value and a low BSA absorption, which means the material has high thrombotic effect. Considering Table 1 and Figure 1a, the EGDMA-based polymer shows higher BCI and lower protein absorption values than TMPTA (Figure 1b). Among all six samples, EGDMA (1:1) polymer gave the best blood compatibility. Therefore, it was concluded that EGDMA-based SAP possesses better biocompatibility than TMPTA-based SAP, which can be further evaluated for in vitro and in vivo studies. Blood biocompatibility studies have shown that EGDMA samples are more biocompatible.

2.1.2. Peptide Release Study. The in vitro analysis was carried out to study the efficiency of the polymer material with peptide. The peptide GHK-Cu was incorporated in the polymer with optimized swelling and biocompatibility. The PASP:AA ratio with best efficiency was observed to be 1:1 in the case of EGDMA as cross-linker and 1:5 in the case of TMPTA as cross-linker. The polymers have been further used for analyzing the release and encapsulation efficiencies of peptide, i.e., GHK-Cu.

Figure 2 explains the GHK-Cu release from polyaspartic acid and acrylic acid polymer with two different cross-linkers (Figure 2a,b). The peptide loading was further confirmed by FTIR and energy-dispersive spectrometry (EDX) analyses to confirm the presence of GHK-Cu Figure 3 (SI3). Among all four models, the peptide release from EGDMA- and TMPTA-based sample follows first-order model (Table 2). The peptide encapsulation was confirmed by FTIR and EDX analyses to confirm the presence of GHK-Cu Figure 4.

The in vitro studies were performed to evaluate the polymer material for drug/peptide-delivery application. The peptide GHK-Cu was used as a drug moiety for the study. The encapsulation efficiency of the polymer/monomer ratio 1:1 with EGDMA as cross-linker has been found to be 78.36%. Drug releases at time intervals of 1, 2, 3, 4, 20, and 24 h are 15.75 ± 2.01, 38.12 ± 3.38, 45.17 ± 4.72, 59.22 ± 2.78, 84.91 ± 4.06, and 92.61 ± 3.10%, respectively. This release of peptide from polymer was further studied using four mathematical models to investigate the mechanism and model of release. The release kinetics from EGDMA-based polymer followed first-order kinetics. The encapsulation efficiency of the polymer with TMPTA was 66.01%. Figure 2b depicts the drug releases (%) at predetermined time intervals of 1, 2, 3, 4, 20 and 24 h to be 30.20 ± 4.14, 42.77 ± 4.89, 48.26 ± 3.25, 55.58 ± 2.75, 69.25 ± 4.89, and 75.32 ± 3.67%, respectively. Furthermore, the release mechanism was evaluated using four models, viz., zero-order, first-order, Korsmeyer Peppas, and Higuchi. This release kinetics of peptide release from TMPTA-based polymer followed the Korsmeyer Peppas model. The peptide encapsulation was confirmed by FTIR and EDX analyses to confirm the presence of GHK-Cu (Figures 3 and 4). The EGDMA-based polymer with 1:1 ratio (PASP:AA) was further evaluated on animal models (in vivo studies) as this polymer showed good peptide release efficiency.

2.2. In Vivo Analysis. The in vitro analysis was performed on two samples, one with EGDMA as cross-linker with polymer/monomer ratio 1:1 and other with TMPTA as cross-linker with polymer/monomer ratio 1:5 represented as

| Table 1. Blood Clotting Index and Protein Adsorption of EGDMA- and TMPTA-Based Polymers |
|-----------------------------------------|----------------|----------------|----------------|
| polymer → PASP:AA (1:5) PASP:AA (1:2) PASP:AA (1:1) |
| parameters ↓ | EGDMA | TMPTA | EGDMA | TMPTA | EGDMA | TMPTA |
| blood clotting index (%) | 58 ± 0.94 | 58 ± 1.02 | 51 ± 1.61 | 21 ± 0.98 | 71 ± 0.76 | 24 ± 0.86 |
| protein adsorption (mg/g) | 30 ± 1.2 | 50 ± 1.01 | 20 ± 0.92 | 37 ± 1.02 | 15 ± 0.88 | 37 ± 1.14 |

Figure 2. Peptide release from (a) EGDMA- and (b) TMPTA-based superabsorbent polymer.

Figure 3. (a) FTIR and (b) EDX spectra of peptide-encapsulated EGDMA-based polymer.
PASP:AA (E) (1:1) and PASP:AA (T) 1:5, respectively. The superabsorbent polymer with EGDMA as cross-linker, which showed better blood compatibility and peptide release results, was further evaluated for in vivo studies. The polymer encapsulated with peptide was used for evaluating the healing potential on the wound bed. The in vivo studies include wound closure, biochemical assay, histopathological examination, and toxicity studies.

The animals were arranged as:
- Group I = control.
- Group II (model) = burned wound (untreated).
- Group III (A) = polymer (polyspartic acid (PASP) and acrylic acid).
- Group IV (A″) = polymer-encapsulated peptide (GHK-Cu).

2.2.1. Wound Closure Study. Wound contraction of a wound shows the effect on wound healing. Wound healing is a complex process that is hindered by different factors. Histopathology images helped us to predict the wound healing process on 8th day and 15th day, as shown in Figure 8. The wound contraction area on application of peptide-encapsulated polymer, polymer, and control was measured on 0th, 8th, and 15th days. On 0th day, wound was created using a thermocouple with 26 mm diameter. On 0th day, wound was created using thermocouple with 26 mm diameter. The wound contraction using peptide-encapsulated polymer showed fastest wound closure compared to polymer and control. On 8th day contraction was observed from 26 to 10 mm with polymer-encapsulated peptide (A″), while for polymer sample (A), the wound area was found to be 15 mm. A wound healing of 61.53% was observed with GHK-Cu-encapsulated polymer and 42.30% with polymer alone. On 15th day, the wound contraction recorded was 4 mm for peptide -encapsulated polymer (A″) and 10 mm for polymer (A). Thus, 84.61 and 61.58% of healing were observed with peptide-encapsulated polymer (A″) and with only polymer, respectively. The release of GHK-Cu on the wound showed remarkable improvement in wound with hair growth observed on wound bed on 15th day. For model (Group II), the wound areas were measured to be 26, 23, and 25 mm (infection) on 0th, 8th, and 15th days, respectively. The wound closure analysis explains that wound treated by the peptide-incorporated polymer (A″) shows fast recovery of epidermal layer on 15th day. The wound contractions (%) from peptide-encapsulated polymer (A″) on 8th day and 15th day were found to be 61.53 and 84.61%, respectively. However, the wound contractions (%) of polyaspartic acid and acrylic acid polymer (A) on 8th and 15th days were found to be 42.30 and 61.53%, respectively. The control (group I) shows unaltered inflammatory cells, epithelial layer, and appendages on healthy skin. The model rat (group II) showed inflammatory cells, ruptured tissues, and proliferation of epidermis with hyperkeratosis on 8th day, whereas on 15th day, it showed decrease in inflammation but no epithelial layer growth. The polyaspartic acid and acrylic acid polymer (group III) were able to accelerate epithelial layer growth on the 15th day and partial epithelialization on 8th day. The peptide-incorporated polymer (group IV) (A″) showed increase in epithelial layer from 8th day to 15th day. Increased angiogenesis, few blood vessels, fibroblast proliferation, and appendages present at 15th day confirm rapid healing of the wound. Histopathological examination also confirmed the fastest healing found in peptide-encapsulated polymer. The toxicity tests showed that polymer with and without peptide did not cause any toxicity to the animal model used (Figure 5).

2.2.2. Biochemical Assay. This study indicates the biochemical changes on the 8th and 15th days. Granulation tissues were collected on the 8th and 15th days for biochemical and antioxidant evaluation. Quantitative estimation of the biochemical parameters of wound tissue is presented in Table 3, whereas graphical representation is shown in Figure 6. The wound contraction is further supported by biochemical assay, histopathology, and toxicity studies. Significant (p < 0.05)
increases of DNA, protein, hexosamine, and hydroxyproline contents observed for peptide-encapsulated polymer (A″) after 8th day were 47, 34, 28, and 29%, respectively, and after 15 days, they were 64, 43, 50, and 38%, in comparison to model (group II). Hydroxyproline is a marker of collagen biosynthesis, whereas hexosamine reflects the effect on granulation tissue as well as stabilization of the collagen molecules by electrostatic and ionic interactions. The estimation of DNA and protein content in wounds indicates the mitotic potential of the wound tissue.

### 2.2.2.1. Antioxidant Evaluation

The activities of the antioxidant enzymes—catalase (CAT), superoxide dismutase (SOD), glutathione, and reactive oxygen species (ROS) groups, were studied as an important indication of treatment process, and the values are reported in Table 4. Enzymatic activities were calculated using skin of all of the six rats in each group taken as an average and including standard deviation.

<table>
<thead>
<tr>
<th>antioxidant activity</th>
<th>model</th>
<th>A</th>
<th>A″</th>
</tr>
</thead>
<tbody>
<tr>
<td>catalase (U/mg protein)</td>
<td>day 8</td>
<td>6.91 ± 0.98</td>
<td>7.96 ± 0.70</td>
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<tr>
<td>glutathione (U/mg protein)</td>
<td>day 8</td>
<td>1.48 ± 0.20</td>
<td>1.63 ± 0.23</td>
</tr>
<tr>
<td>superoxide dismutase (U/mg protein)</td>
<td>day 8</td>
<td>1.32 ± 0.08</td>
<td>1.41 ± 0.08</td>
</tr>
<tr>
<td>reactive oxygen species (free radicals)</td>
<td>day 8</td>
<td>3.50 ± 0.21</td>
<td>3.00 ± 0.20</td>
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Figure 6. Granulation activity of wound by using with (A″) and without (A) peptide-encapsulated polymer.
On introduction of PASP, AA polymer, and polymer with GHK-Cu, these levels further changed, indicating tissue repair and normalization of enzymes and proteins concentration. Significant increase in CAT, SOD, and glutathione and decrease in ROS concentration were observed prominently for groups IV (A″) compared to group II (model). This shows enhanced mitigation of the wound with the polymer with GHK-Cu. A 25−50% improvement in concentration levels was observed with polymer with GHK-Cu on 15th day (Table 4).

Significant increase in the catalase, superoxide dismutase, and glutathione and decrease in ROS concentration were observed prominently for peptide-encapsulated polymer samples. Wound condition, of group II animals (untreated wound), showed reduced levels of CAT, SOD, and glutathione and increased levels of ROS in the case of control samples. The maximum healing effect was seen for polymer with GHK-Cu, which attained the desired values in comparison to model. The wound healing was observed to follow the following trend as: model < PASP:AA < polymer with GHK-Cu (Figure 7).

2.2.3. Histopathological Examination. Wound contraction in a wound shows the effect on wound healing. Wound healing is a complex process that is hindered by different factors. Histopathology images helped us in predicting the wound healing process. The wound contraction areas on application of peptide-encapsulated polymer, polymer, and control were...
measured on 0th, 8th, and 15th days as shown in Figure 8. On 0th day, wound was created using thermocouple with 26 mm diameter. The control (group I) shows unaltered inflammatory cells, epithelial layer, and appendages on healthy skin.

| Table 5. Toxicity Levels in Rat after Using Polymeric Material for Wound Healing |
|-------------------------------------------------|----------|----------|----------|----------|
|                           | control   | model    | A        | A*       |
| SGOT (U/L) reference range (45.7–80.8)       | 45 ± 2.43 | 48 ± 2.84 | 65 ± 2.55 | 50 ± 3.01 |
| day 8                              | 46 ± 2.01 | 50 ± 2.76 | 61 ± 2.65 | 51 ± 2.98 |
| day 15                             | 18 ± 2.11 | 20 ± 2.21 | 25 ± 3.07 | 28 ± 3.11 |
| SGPT (U/L) reference range (17.5–30.2)      | 19 ± 2.15 | 27 ± 2.78 | 26 ± 2.98 | 29 ± 2.88 |
| day 8                              | 0.21 ± 0.01 | 0.28 ± 0.02 | 0.40 ± 0.03 | 0.30 ± 0.02 |
| day 15                             | 0.22 ± 0.02 | 0.29 ± 0.01 | 0.41 ± 0.03 | 0.29 ± 0.03 |
| ALP (U/L) reference range (0.2–0.8)       | 16.00 ± 1.67 | 16.75 ± 1.37 | 19.34 ± 1.84 | 18.33 ± 1.99 |
| day 8                              | 17.10 ± 2.01 | 16.18 ± 1.60 | 20.56 ± 1.92 | 19.23 ± 2.06 |
| day 15                             | 61 ± 3.87  | 64 ± 4.30  | 82 ± 3.77  | 67 ± 3.60  |
| blood urea nitrogen (mg/dL) reference range (56.8–128) | 62 ± 3.43 | 65 ± 3.98 | 85 ± 4.22 | 68 ± 4.01 |
| day 8                              | 0.23 ± 0.02 | 0.30 ± 0.03 | 0.35 ± 0.02 | 0.27 ± 0.02 |
| day 15                             | 0.25 ± 0.03 | 0.29 ± 0.04 | 0.38 ± 0.02 | 0.28 ± 0.03 |
| Bilirubin (mg/dL) reference range (0.2–0.55) | 0.23 ± 0.02 | 0.30 ± 0.03 | 0.35 ± 0.02 | 0.27 ± 0.02 |
| day 15                             | 0.25 ± 0.03 | 0.29 ± 0.04 | 0.38 ± 0.02 | 0.28 ± 0.03 |

**Figure 9.** Toxicity levels found in rat after using polymeric material for wound healing.
model (Group II), the wound areas were 26, 23, and 25 mm (infection) on 0th, 8th, and 15th days, respectively. The model rat (group II) showed inflammatory cells, ruptured tissues, and proliferation of epidermis with hyperkeratosis on 8th day, whereas on 15th day, there was decrease in inflammation but no epithelial layer growth. On 8th day, contraction was observed from 26 to 10 mm with polymer-encapsulated peptide (A°), while for polymer sample (A), the wound area was found to be 15 mm. A wound healing of 61.53% was observed with GHK-Cu-encapsulated polymer and 42.30% with polymer alone. On 15th day, the wound contraction recorded was 4 mm for peptide-encapsulated polymer (A°) and 10 mm for polymer (A). Thus, 84.61 and 61.58% of healing were observed with peptide-encapsulated polymer (A°) and with only polymer, respectively. The polyaspartic acid and acrylic acid polymer (group III) was able to accelerate epithelial layer growth on 15th day and partial epithelialization on 8th day. The peptide-incorporated polymer (group IV) (A°) showed increase in epithelial layer from 8th day to 15th day. Increased angiogenesis, few blood vessels, fibroblast proliferation, and appendages present at 15th day confirm rapid healing of the wound. The release of GHK-Cu on the wound showed remarkable improvement in wound with hair growth observed on wound bed on 15th day. Histopathological examination also confirmed the fastest healing found in peptide-encapsulated polymer. The wound contraction using peptide-encapsulated polymer showed fastest wound closure than polymer and control. The wound closure analysis explains that wound treated by the peptide-incorporated polymer (A°) shows fast recovery of epidermal layer on 15th day.

2.2.4. Toxicity Tests. The toxicity tests were performed to analyze the toxicity effect of the polymeric material and peptide-encapsulated polymer material on the liver and kidney. The tests were performed on serum separated from blood. The procedure for these tests is explained in detail in the Supporting Information (SI2). There is a standard reference range for all mentioned toxicity tests in Table S. From Table S, it is clear that all toxicity tests performed were in series. The control and model animals did not show any toxicity due to application of the polymeric material (Figure 9).

3. CONCLUSIONS

To conclude, we have synthesized biocompatible polyaspartic acid-based superabsorbent polymer. This synthesized superabsorbent was loaded with a peptide possessing healing properties. Peptide-encapsulated polymer (A°) is more efficient for wound healing compared to polymers alone (A). GHK-Cu has been found to produce effective wound healing. The remarkable healing using peptide-encapsulated polymer was confirmed by wound closure, biochemical assay, histopathological examination, and toxicity studies.

4. EXPERIMENTAL SECTION

4.1. Preparation of Polyaspartic Acid- and Acrylic Acid-Based Polymer. The synthesis of polyaspartic acid- and acrylic acid-based superabsorbent polymer has been discussed in detail in our previous work. Different polymers were synthesized by varying the ratio of polyaspartic acid (PASP), acrylic acid (AA) with initiator, and cross-linker (EGDMA and TMPTA) at constant temperature range 80–85 °C. The polymer with the highest swelling in water was further studied as explained in our last research. The synthesized polymer was placed in dialysis bag (mesh size) 10 kDa in water for 7 days to remove the unreacted monomer.

4.2. In Vitro Studies. 4.2.1. In Vitro Blood Compatibility of the Synthesized Polymers. 4.2.1.1. Bovine Serum Albumin (BSA) Adsorption/Protein Adsorption. The amount of protein adsorbed (mg/g) was calculated using the following equation

\[ \text{adsorbed BSA} = \frac{\left( C_0 - C_f \right) \times V}{W} \]  

where

- \( C_0 \) = BSA concentration before adsorption (mg/mL).
- \( C_f \) = BSA concentration after adsorption (mg/mL).
- \( V \) = weight of the swollen hydrogel (g).
- \( W \) = volume of BSA solution used (mL).

All measurements were done in triplicate and the averages of these values together with their standard deviation have been reported.

4.2.1.2. Blood Clotting Test. The blood clotting index (BCI) has been calculated by using the following equation

\[ \text{BCI} = \frac{\text{OD}_t}{\text{OD}_c} \times 100 \]  

where

- \( \text{OD}_t \) = optical density of the test sample.
- \( \text{OD}_c \) = optical density of the control sample.

All measurements were done in triplicate and have been reported taking the average and calculating the standard deviation.

4.2.2. Peptide Encapsulation. The encapsulation efficiency was measured by comparing the UV absorption spectra of peptide-loaded polymeric sample using calibration curve of peptide.

The encapsulation efficiency was calculated using the following equation

\[ \text{encapsulation efficiency} = \frac{\text{amount of drug added} - \text{amount of free drug}}{\text{amount of drug added}} \times 100 \]  

4.2.3. Peptide Release Studies. The amount of peptide release was measured at 605 nm using UV spectrophotometer at predetermined time intervals, i.e., 1, 2, 3, 4, and 24 h. Aliquot (3 mL) was used for absorbance measurement. Volume of the solution was maintained by returning back aliquots after measurement.

4.3. In Vivo Studies. 4.3.1. Animal Grouping and Dosing. A total of 24 wistar rats of weight 220 ± 20 g were used. The animal experiments were performed according to ethical approval (949/IAEC/16) and guidelines of All India Institute of Medical Sciences, New Delhi, India. The animals were separately kept in clean cages and were divided into three groups

(i) Group I = control.
(ii) Group II (model) = burned wound (untreated).
(iii) Group III (A) = polymer (polyaspartic acid (PASP) and acrylic acid).
(iv) Group IV (A°) = polymer-encapsulated peptide (GHK-Cu).

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The in vivo experiments were performed in triplicate with six animals in each group II, III, and IV and three animals in control group.

4.3.2. Excision Burn Wound Model. Wistar rats (220 ± 20 g) were anesthetized, and dorsal hairs were shaved and sanitized using ethanol. A cylindrical stainless rod of 35 mm diameter with insulated rubber handle was used for burns. The temperature was maintained by the instrument according to the feed. The temperature for second-degree burn was 100 °C. The duration of burn on wistar rat was 30 s. After creating the burn wound, the polymeric materials were applied on the wound bed.46

4.3.3. Measurement of Wound Area. Wound contraction was measured on 8th and 15th days using a vernier caliper. The comparative healing rate was evaluated by comparing mean wound contraction in comparison to control.43 The percentage wound contraction was calculated using the following equation

\[ \text{wound contraction} = \left( \frac{W_n - W_0}{W_0} \right) \times 100 \]  

where

\[ W_0 = \text{wound area on zero day} \]
\[ W_n = \text{wound area on nth day (n = 8, 15)} \]

4.3.4. Biochemical Analysis. The tissues were collected on 8th and 15th days for biochemical assay. The granulation tissues were assessed for DNA, protein, hydroxyproline, and hexosamine.44 The antioxidant levels in tissue were evaluated by studying the levels of glutathione, catalase, superoxide dismutase, and reactive oxygen species. All methods are described in the Supporting Information (S1).

4.3.5. Histopathological Evaluation. For histopathological evaluation, the animals were sacrificed on 8th and 15th days as wound tissues were fixed with 10% formalin solution. Tissue blocks in paraffin were prepared and sectioned using Microtome (RM 2235, Germany). The sectioned tissues were collected on glass slides, stained using hematoxylin and eosins, and evaluated using a microscope. Hematoxylin and Eosin stains were used to visualize and differentiate between control, model, polymer, and peptide-incorporated polymer tissues. All morphometric parameters were recorded using an image analyzer (Olympus microscope BX61, Japan). The epidermal and dermal regions were evaluated. Digital photomicrographs were captured and wounds were evaluated for new blood capillaries formation and regeneration.45

4.3.6. Toxicity Tests. The toxicity tests were performed to investigate the effect of polymer and peptide-incorporated polymer on liver and kidney. Blood samples were collected from cardiac puncture of rats. The serum separated from blood was used for performing liver and kidney tests. These liver tests include SGOT, SGPT, ALP, and Bilirubin, whereas blood urea nitrogen and creatinine tests are to evaluate the toxicity content present in rat.46-48 The detailed explanation of toxicity tests is presented in the Supporting Information (S1).

4.3.7. Statistical Analysis. All of the values are presented as mean ± standard deviation using GraphPad Prism software. Statistical analysis were performed using t-test. P values < 0.005 were considered significant.

**REFERENCES**


