Materials Letters 228 (2018) 375–378

Contents lists available at ScienceDirect

Materials Letters

journal homepage: www.elsevier.com/locate/mlblue

# Crosslinked gelatin hydrogels as carriers for controlled heparin release

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ARTICLE INFO

*Article history:*
Received 1 March 2018
Received in revised form 25 May 2018
Accepted 13 June 2018

*Keywords:*
Gelatin hydrogel
Crosslinking
Heparin
Therapeutic agent
Anticoagulant
Platelets adhesion

ABSTRACT

The application of heparin as anticoagulant, anti-inflammatory and growth factor regulating agent is currently limited by its narrow therapeutic window. Here, we describe the use of chemically crosslinked gelatin hydrogels as delivery platform to achieve the control of heparin release over time. Different hydrogel formulations and two strategies for heparin loading were tested. The synergic electrostatic interactions between heparin and gelatin hydrogels resulted in a sustained release until 60 h, demonstrated by toluidine blue tests. Platelets adhesion was significantly reduced in heparin-loaded hydrogels, thus proving good heparin bioactivity after processing. Our heparin-loaded hydrogels represent a possible valid option to develop coating for catheters and cardiovascular devices, or skin dressings.



## 1. Introduction

Unfractioned heparin is a well-established anticoagulant used to treat thrombotic disorders [1], stabilize growth factors [2], and develop anti-inflammatory and tumor-inhibiting agents [3,4]. However, its narrow therapeutic window requires careful dosing and monitoring to avoid bleeding [1,4]. Despite alternative therapies (e.g., low molecular weight heparin, direct thrombin inhibitors) [5], unfractioned heparin is still widely used in coating technologies (e.g., catheters) and cosmetics [6]. In addition, the engineering of the beneficial properties of heparin is a growing branch in biomaterials science and novel approaches have been recently proposed [7–9]. Controlled heparin release still remains an open challenge [10]. A possible solution is the design of a proper reservoir that carries the therapeutic agent and slowly releases it *in situ*. Hydrogels are promising drug delivery platforms due to their relatively low cost, versatile processing, high water content and tailorability of the hydrogel physico-chemical properties [11,12]. In particular, gelatin, a partially denatured derivative of collagen, has outstanding biocompatibility, low cost and processability. Also, compared to collagen, gelatin presents lower thrombogenicity and host immune response [11].

Here, we investigate the possible use of crosslinked gelatin hydrogels as carriers for heparin, testing different hydrogels prepared using two loading methods. The release of heparin was quantified by a toluidine colorimetric assay. *In vitro* tests were performed to check platelet adhesion.

## 2. Materials and methods

### 2.1. Gelatin hydrogels preparation

Chemically crosslinked gelatin hydrogels were prepared at 50 °C by mixing type A gelatin from porcine skin (isoelectric point, pI: 7.0–9.0, Sigma-Aldrich), *N,N′*-methylenebis(acrylamide) as crosslinker (MBA, Sigma-Aldrich) and triethylamine as activation agent (TEA, Sigma-Aldrich), following an optimized protocol [13] (Table 1). Three formulations were synthesized by varying gelatin concentration (15 or 20% w/v) and crosslinking stoichiometry (MBA:gelatin amino groups = 0.5:1 or 1:1). Heparin-loaded hydrogels were then prepared using two loading methods (Table 1): hydrogels synthesized in presence of heparin (INP) and loaded with heparin by absorption (LOA). Samples without heparin (GEL) were used as control. Consistently with routinary clinical procedures [14], INP and LOA samples were loaded with 2.5 mg heparin (sodium heparate, Pharmaceuticals Partners of Canada) per sample (diameter Ø = 6 mm). For INP, the heparin solution was added in the proper amount during synthesis, together with

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https://doi.org/10.1016/j.matlet.2018.06.047
0167-577X/

**Table 1**
Hydrogel formulations and heparin loading methods. The names are composed of gelatin concentration + type of sample + reaction stoichiometry.

| Name | Gelatin in water [wt. %] | TEA [μL] | MBA [mg] | Heparin during synthesis | Heparin absorption |
|---|---|---|---|---|---|
| 15GEL05 | 15 | 60 | 23.3 | N | N |
| 15GEL1 | | | 46.6 | | |
| 20GEL1 | 20 | 80 | | | |
| 15LOA05 | 15 | 60 | 23.3 | N | Y |
| 15LOA1 | | | 46.6 | | |
| 20LOA1 | 20 | 80 | | | |
| 15INP05 | 15 | 60 | 23.3 | Y | N |
| 15INP1 | | | 46.6 | | |
| 20INP1 | 20 | 80 | | | |

MBA; for LOA, heparin was loaded by swelling the xerogel overnight at 37 °C in 1 mL Phosphate Buffered Saline (PBS, LifeTechnologies) solution containing 250 units/mL heparin sodium salt. The PBS amount was previously optimized (unpublished data) to guarantee the complete absorption of loading medium (i.e., all the heparin as well). Samples were then dehydrated in ethanol before further characterization.

Hydrogels produced with this protocol dissolve in water within a finely tunable period of 2–30 days. Degradation and mechanical properties (E = 20–200 kPa, $\sigma_{max}$ = 20–100 kPa) depend on the specific formulation (unpublished data).

### 2.2. Heparin release

A toluidine blue assay protocol [15] was adapted to quantify the release of heparin from the hydrogels. Swollen hydrogel specimens were immersed in 10 mL NaCl 0.2% w/v saline [15]. Aliquots were collected at selected time-points (t = 2, 12, 60 h) and mixed with saline and 1 M HCl at a 10:1 ratio, plus 0.01% w/v toluidine. These solutions were then further mixed 1:1 with hexane (Sigma-Aldrich) to separate three phases: hexane, free toluidine in water and toluidine-heparin complexes at the interface. The organic supernatant was removed and water solutions were analyzed by UV–Vis-NIR spectrophotometry (λ = 630 nm, Agilent Technologies), calibrated by a standard curve of serial dilution of heparin (0–200 μg/mL range). A release efficiency index (REI) was defined as ratio of released ($Hp_i$) to theoretically loaded heparin (i.e., 2500 μg):

$$REI[\%] = \frac{\sum Hp_i[\mu g]}{2500[\mu g]} \times 100 \quad (1)$$

### 2.3. In vitro platelet adhesion

Platelet adhesion was evaluated on PBS-preconditioned samples (24 h, n = 3 per hydrogel type), subsequently immersed in fresh human blood obtained from a healthy volunteer after written informed consent. This study was performed following a protocol [16] approved by the human and animal ethical committees of the Montreal Heart Institute (see Supplementary data, Project #2001-5, 406, (01-069)-PSGL-1, FWA00003235). No additional anticoagulant was used. Samples were kept under shear stress on a lab rocker at 80 tilts per minute for 30 min, then stained with 200 μg/mL Alexa Fluor 647 anti-human CD61 antibody (BioLegend), fixed with paraformaldehyde (Sigma-Aldrich) and observed by confocal microscopy ($\lambda_{exc}$ = 633 nm, $\lambda_{em}$ = 647 nm, LSM 510, Zeiss). The images (n = 3 for each tested condition) were used to quantitatively evaluate platelets number on different hydrogels samples by pixel counting (ImageJ software, one white pixel = one platelet).

### 2.4. Statistical analysis

Data are reported as mean ± standard deviation; differences were verified by One-Way ANOVA test, considering $p < 0.05$ as statistically significant.

## 3. Results and discussion

### 3.1. Heparin release

The absorbance values of GEL formulations are comparable to a toluidine control solution without heparin ($p > 0.05$), confirming negligible interactions between gelatin and toluidine. A uniform release of heparin, without initial burst, was observed for heparin released from INP (Fig. 1A) and LOA (Fig. 1B) samples.

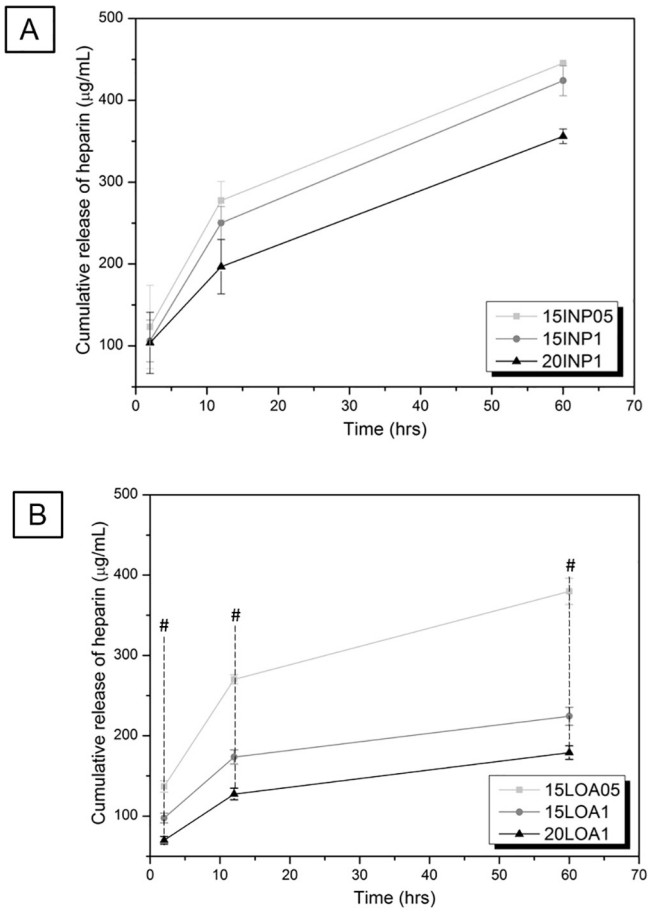


**Fig. 1.** Cumulative release of heparin from (A) LOA and (B) INP hydrogels (*$p < 0.05$).