Crosslinked gelatin hydrogels as carriers for controlled heparin release

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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

Unfractionated 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.

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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, σmax = 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 (Hpi) to theoretically loaded heparin (i.e., 2500 µg):

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REI[\%] = \frac{\sum Hpi[\mu g]}{2500[\mu g]} \times 100
\] (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 (λexc = 633 nm, λem = 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).](image-url)
Different release trends characterize LOA and INP samples. LOA samples show a more accentuated plateau of release and release profiles significantly differ depending on hydrogel formulation; in particular, 15LOA05 releases higher quantities of heparin \( (p < 0.05) \). On the contrary, release trends from INP samples are steady over time, with no statistically difference between the considered formulations \( (p > 0.05) \). The different behavior of LOA and INP samples can be related to a deeper inclusion of heparin in INP samples, released during the hydrogel degradation. For both the tested strategies, the absence of burst release suggests the instauration of electrostatic interactions between gelatin and heparin, characterized by a high number of negative charges due to its sulfate residues \([17]\) interacting with cationic charges of type A gelatin at neutral pH. In particular, previous studies \([18,19]\) identify positively charged amino groups \((-\text{NH}_3^+)\) on gelatin as crucial binding sites for heparin. These can be found primarily on the side chain of lysine and are fully protonated at neutral pH \( (pK_a = 10.53) \) \([18,19]\). Comparing REI values (Fig. 2), 15INP1 and 20INP1 samples have significantly higher REI than the respective LOA samples \( (p < 0.05) \). Moreover, 15INP05 has the highest REI. However, it was characterized by a faster degradation (i.e., dissolved in two days, unpublished data), compared to 15GEL05, stable for several weeks, probably due to a shielding of crosslinking sites by heparin \([20,21]\). The different degradation rates of the considered hydrogels make them adequate for different applications depending on the requested heparin release profile. Higher heparin loading efficiencies were reported for chitosan-based systems \([22,23]\), but these systems sustain heparin release for a shorter time (i.e., max 8 h), compared to crosslinked gelatin hydrogels. Kuo et al. \([24]\) obtained a uniform release with 14% efficiency via a polylactide-co-glycolide (PLGA) carrier, but a significantly higher initial heparin expense was required. Hence, our gelatin-heparin hydrogels, in particular INP formulations, offer a better compromise between efficiency, release profile and release time window, compared to other heparin-delivering systems.

### 3.2. In vitro platelet adhesion

Representative confocal images of platelets adhered on 15GEL1, 15LOA1 and 15INP1 samples are shown in Fig. 3a. White dots represent platelets stained with antibodies, while the background black field is the substrate. Higher platelet adhesion can be qualitatively observed as higher density of white pixels on GEL compared to LOA and INP samples. The platelet count confirms
that the number of platelets adhered on the heparinized gel (i.e., INP and LOA) is significantly lower (p < 0.05) than that on GEL samples (Fig. 3b). A reduction of 90% in platelets adhesion can be observed, without substantial difference between the two considered loading strategies.

All samples reduced platelet adhesion similarly, apart from 15LOA1, which gave a reduction that set slightly above the other formulations (p > 0.05). Lower adhesion on heparin-loaded samples is probably due to a combination of the anticoagulant effect of heparin and its ability of increasing the gelatin hydrophilicity (see Supplementary data for contact angle measurements). In fact, hydrophilicity affects platelets adhesion, since platelets have higher affinity to hydrophobic surfaces [25,26]. These promising results require further in vitro tests to deeper investigate platelet activation.

4. Conclusion

Heparin was successfully incorporated into crosslinked gelatin hydrogel and its release over 60 h was evaluated. The incorporation of heparin during the synthesis (i.e., INP) obtained the best release over time. A strong inhibitory effect on platelets adhesion was preliminarily assessed, suggesting intact heparin biological activity. The proposed delivery approach is characterized by competitive heparin release efficiency compared to analogue systems in literature. These promising findings incentivize further in vitro tests to quantify the thrombogenicity of gelatin and platelet activation. After that, heparin-loaded crosslinked gelatin hydrogels with the appropriate degradation rate and heparin release could find applications in the development of coatings for catheters and cardiovascular devices, or as skin dressing components.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.matlet.2018.06.047.

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