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An in situ cross-linking hybrid hydrogel for controlled release of proteins

Hila Epstein-Barash^{1,2}, Cristina Stefanescu¹, and Daniel S. Kohane¹

¹Laboratory for Biomaterials and Drug Delivery, Department of Anesthesiology, Division of Critical Care Medicine, Children's Hospital, Harvard Medical School, 300 Longwood Ave., Boston, MA 02115

²Harvard-Massachusetts Institute of Technology Division of Health Sciences and Technology, Cambridge, MA, USA 02139

Abstract

There is a clear need for methods of providing safe controlled release of therapeutic proteins, either to achieve and maintain high local protein concentrations, or for sustained systemic delivery. Here we have developed a protein delivery system combining in situ cross-linkable polysaccharide hydrogels with gelatin. This formulation is injectable, easy to apply, and obviates the need for organic solvents or potentially toxic cross-linking agents in the formulation process. The cross-linked polysaccharides themselves (comprising hyaluronic acid, dextran, and/or carboxymethylcellulose) provided prolonged release of fluorescently-labeled albumin (FITC-albumin). The duration of release was markedly extended by the incorporation of gelatin into the formulation: FITC-albumin and IL-2 were released over the course of more than three weeks. The IL-2 maintained > 70% activity throughout that time. Gelatin also accelerated the gelation time of the hydrogels, and reduced their swelling in phosphate buffered saline. The composite hydrogel (dextran-carboxymethylcellulose-gelatin) showed minimal cytotoxicity *in vitro*, and benign tissue reaction after subcutaneous injection in rats.

Keywords

controlled release; protein; gelatin; drug delivery; hydrogel

1. Introduction

The proliferation of protein-based therapeutics has created broad interest in the development of controlled release technologies for proteins, either to achieve and maintain high local protein concentrations, or for sustained systemic delivery. Proteins can benefit greatly from controlled release systems, to overcome problems with bioavailability, and short plasma half-lives [1, 2]. Therefore, a number of controlled release systems, including osmotic

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Corresponding author: Daniel S. Kohane, M.D., Ph.D., Laboratory for Biomaterials and Drug Delivery, Department of Anesthesiology, Division of Critical Care Medicine, Children's Hospital, Harvard Medical School, 300 Longwood Ave, Boston, MA 02115 Phone: 617-355-7327, FAX: 617-730-0453, daniel.kohane@childrens.harvard.edu.

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pumps [3, 4], Pluronic gels [5], gelatin [6], collagen [7, 8] and biodegradable polymeric particles [9] have been used to prolong the systemic serum presence of proteins.

Recently, there has been increasing interest in highly localized protein delivery over extended periods, particularly since systemic distribution of many proteins can create toxic side effects. For example, polymer-based microsphere formulations have been developed to provide sustained, localized, cytokine delivery [9]. Hydrogels can be excellent candidates for protein delivery [6, 10] since they may not have the pro-inflammatory effects of polymeric systems [11] and tissue reaction to them tends to be benign [12, 13]. Furthermore, unlike polymeric systems there may be no need for surfactants, organic phases or sonication which can reduce the biologic efficacy of the protein [2, 14]. Protein-based hydrogels may be able to control the rate of release of proteins drugs through electrostatic interactions as well as through degradation, resulting in quite extended release profiles, as was the case with gelatin-based systems [2, 14]. Disadvantages of the gelatin hydrogels included the fact that formation required a cross-linker such as glutaraldehyde [15], and that as a consequence, the gels had to be cast in advance. While they could be produced as microspheres, that still does not allow the flexibility upon application of in situ cross-linking hydrogels in terms of reliably coating complex surfaces (such as the peritoneal cavity, or specific portions thereof) in an ad hoc manner during procedures [16].

We have previously shown that hyaluronic acid hydrogels [17] that cross-link *in situ* by hydrazone bond formation have excellent biocompatibility in the peritoneum – a relatively sensitive anatomic location [18] – and that when optimized those hydrogels can release a protein such as tissue plasminogen activator (tPA) with great effectiveness in preventing adhesions [19]. Although the released tPA was biologically effective, approximately 80 percent of it was released within two days [19].

Here we have hypothesized that combining in situ cross-linkable polysaccharide hydrogels with gelatin would provide the ease of application and biocompatibility of in situ cross-linking hydrogels with the prolonged protein release of gelatin-based systems, without needing surfactants and toxic cross-linkers.

2. Materials and Methods

2.1. Materials

Carboxymethylcellulose (CMC, medium viscosity), dextran (100 kDa), adipic dihydrazide (ADH), 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC), hydroxybenzotriazole (HOBt), sodium periodate, ethylene glycol, tert-butyl carbazate (t-BC), sodium bicarbonate, sodium chloride, acetic acid, albumin-FITC, and acidic gelatin were from Sigma (St. Louis, MO). Hyaluronic acids (HA, Mw = 490 kDa and 1.4 MDa), were purchased from Genzyme (Cambridge, MA).

2.2. Preparation and characterization of hydrogels

Several hydrogels were investigated. Their compositions are abbreviated as follows: hyaluronic acid, HA; carboxymethylcellulose, CMC; aldehyde modification, -CHO; adipic hydrazide modification, -ADH. Cross-linked polysaccharides are denoted throughout by hyphenated abbreviations, e.g. HA-CMC, with subscripts denoting the %w/v of each where appropriate. The first polysaccharide named is the aldehyde, the second the hydrazide. In some experiments, where more than one component (hydrogel precursor or protein) is combined in a given syringe, the contents of that syringe are denoted by inclusion in square brackets; the other components are therefore in the other syringe. All hydrogel components in a given syringe have the same modification (aldehyde or hydrazide).

Three hydrogels were tested: HA-CMC, dextran-CMC, and CMC-CMC. A polymer concentration of 6% for all dextran-CHO and 2.5% for all of the modified hyaluronic acid and carboxymethylcellulose components was chosen based on previous work; these were concentrations that allowed rapid gel formation [20, 21]. Dextran-CHO, CMC-CHO and HA-CHO were prepared as described [21]. In brief, 1.5 g dextran/HA/CMC were dissolved in 150 mL of distilled water overnight, to which 802.1 mg of sodium periodate was added and stirred for 2 hours. 400 μ L of ethylene glycol was added at 2 h to stop the reaction and the mixture was left to stir for an additional 1 h. CMC-ADH was prepared as described [21]. In brief, 0.5 g CMC was dissolved in 100 mL of distilled water, and reacted with 1.5 g of ADH in the presence of 240 mg EDC and 240 mg HOBt at pH 6.8 overnight at room temperature. The products were purified by exhaustive dialysis for 3 days, and then lyophilized. The purified product was freeze-dried and kept at 4 °C.

Preparation of hydrogel disks: the hydrogels were formed using a double-barreled syringe (Baxter: Deerfield, IL). A first syringe contained 1 mL of 2.5% CMC-ADH solution in double distilled water (DDW). A second syringe contained 6% dextran-CHO or 2.5% HA-CHO, or 2.5% CMC-CHO in DDW. The final concentration of hydrogels were kept constant and the total volume kept at 1 mL.

In selected experiments (Table 1) gelatin was added to the polysaccharide solution in one of the two syringes of the double-barreled syringe system. The maximal concentration of gelatin that allowed injectability was 2% in a given syringe (which would yield a final concentration of 1% in the cross-linked hydrogel after combination with an equal volume of gelatin-free polysaccharide). Gelatin was usually added to the aldehyde as it was less viscous than the hydrazide. The aldehyde was dissolved fully, then 2% w/v gelatin was added as a powder and stirred for 2hr. In selected experiments, the gelatin was added to the hydrazide polysaccharide. In those cases, the gelatin and the hydrazide were added as dry powders to phosphate-buffered saline (PBS) and vortexed for 2 hr then stirred overnight.

The two solutions were merged by injection into a rubber mold sandwiched between two slide glasses, forming a hydrogel. The diameters and the thicknesses of the hydrogels were 1.2 cm and 3.5 mm, respectively.

The gelation time was considered to be the time required for the solution to form a globule that separated from the bottom of the dish, as described [22]. The swelling ratio following incubation in PBS was calculated as the weight at a given time point (3 days and 5 weeks) divided by the initial weight of the hydrogel, as described [20].

The morphology of lyophilized hydrogels with and without gelatin was examined by scanning electron microscopy (SEM). Hydrogels were lyophilized then sputter-coated with palladium and gold (150 Å thick) and imaged with a scanning electron microscope (JEOL JSM 6320, JEOL USA, Inc., Peabody, MA). Subsequently, the hydrogels were fractured and coated again to image their internal structure.

2.3. In vitro release kinetics and gel-degradation profiles

Release from various gels was evaluated using FITC-albumin as a model drug. Solutions of modified polysaccharides were prepared in PBS at different concentrations (Table 1). CMC was used at a fixed concentration of 2.5%. FITC-albumin (10 mg/mL) was added to selected solutions (Table 1). For the hybrid gels that contained gelatin, the precursor polymer was weighed as a powder and combined with gelatin powder, dissolved in DDW and lyophilized. The lyophilized material was re-dissolved by drop-wise addition of albumin-FITC solution. Disk-shaped gels were prepared in a rubber mold sandwiched between two slide glasses. Discs were weighed and placed in a Transwell (Corning) perforated with ten 22-G needle

holes to allow media to flow freely into the wells of an underlying 24-well plate. Two milliliters of PBS were added to each Transwell, which was incubated at 37 °C on an orbital shaker. The medium was completely replaced at predetermined time intervals. The FITC-albumin content in media was analyzed by measuring absorbance at 495 nm. In a separate experiment, the wet mass of each gel was weighed after blotting at each time point. Elution of IL-2 was measured with an ELISA kit (R&D Systems, MN, USA). At each time point, the release media containing IL-2 were frozen; activity (Sec. 2.4) was assayed once all samples were collected.

2.4. Bioactivity assay for IL-2

The bioactivity of the released IL-2 was determined in an IL-2-dependent murine cytotoxic T cell line CTLL-2 [23]. In brief, CTLL-2 cells were incubated with known masses of IL-2 in the release media from the experiments described above; samples from 2, 6, 8, 24, 120, 240 and 646 h were analyzed. Twenty-four hours afterwards, cell proliferation was assayed by the MTT test (a colorimetric assay that measures metabolic activity and is widely used to study cell proliferation, cytotoxicity, etc., described in detail below). Results are expressed as a percentage of the activity achieved by the same mass of an IL-2 standard.

2.5. Cytotoxicity assay

Hybrid hydrogels were investigated in a human mesothelial cell line (CRL-9444: American Type Culture Collection (ATCC), Manassas, VA) and macrophage cell line J774.A1 (TIB-67TM: ATCC) using the MTT assay (MTT kit, Promega G4100 Madison, WI). In brief, mesothelial cells were grown and maintained in complete growth medium (Medium199 with Earle's BSS, 0.75 mM L-glutamine and 1.25 g/L sodium bicarbonate supplemented with 3.3 nM epidermal growth factor, 400 nM hydrocortisone, 870 nM insulin, 20 mM HEPES and 10% fetal bovine serum (Gibco)) at 37 °C in 5% CO₂. Macrophages were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, CA) with 10% fetal bovine serum. Hydrogels discs with gelatin were placed in each well and floated in the culture medium 24 h after seeding the cells. MTT assays were performed after 48, 72 and 96 hr, then every 7 days for up to 3 weeks after adding the hydrogels, for macrophages and mesothelial cells. Results were normalized to cells cultured without test compounds.

2.6. Subcutaneous application

Animals were cared for in compliance with protocols approved by the Massachusetts Institute of Technology Committee on Animal Care, in conformity with the NIH guidelines for the care and use of laboratory animals (NIH publication #85-23, revised 1985).

Male CD-1 mice (Charles River Laboratories, Wilmington, MA), (20–28 g) were anesthetized with 2–3% isoflurane in oxygen. 0.1 mL of hydrogel was injected from a double-barreled syringe (0.05 ml of 2.5% CMC- ADH in one syringe, 0.05 mL of 6% dextran-CHO with or without gelatin, in the other) subcutaneously (SC). Animals were euthanized 7 or 21 days after injection. Tissues were harvested and processed into hematoxylin-eosin sections by standard techniques.

2.7. Statistics

Data are presented as means $\pm$ standard deviations ($n = 4$ in release kinetics, cell work, and $n = 6$ for *in vivo* studies). To take multiple comparisons into account, all statistical comparisons were done with the Tukey-Kramer test, using InStat software (GraphPad, San Diego CA). A P -value <0.05 was considered to denote statistical significance.

3. Results

3.1. Hydrogel preparation and characterization

Polysaccharides were modified so as to bear either aldehyde (-CHO) or adipic anhydride (-ADH) functionalities, which would cross-link when mixed, forming hydrazone bonds [17]. Carboxymethylcellulose (CMC), hyaluronic acid (HA) and dextran were modified with -CHO; only CMC was ADH-modified. (Please see Methods for the system of abbreviations for cross-linked hydrogels.) This selection of polymers, polymer concentrations, and modifications was based on our previous work [16, 20, 21, 24]. The various CHO-polysaccharides were combined with CMC-ADH by placing them in separate syringes in a double-barreled syringe (Table 1). Gelatin and/or FITC-albumin were incorporated by inclusion in one syringe or the other. Gelatin accelerated gelation of all hydrogels, and reduced swelling to varying degrees (Fig.1). Hydrogels containing gelatin were also easier to separate from their molds, partly because they were less fragile. By SEM (Fig. 2), lyophilized dextran₆-CMC gels displayed a porous network, typical of cross-linked hydrogels. The porosity of the hydrogel was reduced by addition of gelatin: pores could be observed in the gelatin hydrogels but they were very few and only in the part of the gels shown, while pores in the hydrogel without gelatin were distributed throughout the entire disk in a much more homogeneous pattern.

3.2. Release kinetics of FITC-albumin from cross-linked hydrogels

To identify the hydrogel combination with the slowest release of a model protein (FITC-albumin, Table 1), in vitro release kinetics were performed in phosphate buffered saline (PBS) at 37 °C. All 3 combinations showed burst release, with a significant amount coming out in the first 10 min after placing the gels in PBS (Fig. 3A). The burst was significantly smaller for the gels that contained dextran ($p < 0.001$) - the percentages of total FITC-albumin released over 10 min were CMC_{2.5}-CMC_{2.5}: 26%, HA_{2.5}-CMC_{2.5}: 25%, dextran₆-CMC_{2.5}: 3.4% (Fig. 3A).

Combining different hydrogel precursors (Fig 3B) resulted in intermediate release kinetics. For example, a gel formed by combining a mixture of 3% dextran-CHO and 1.25% CMC-CHO with 2.5% CMC-ADH ([dextran₃+ CMC_{1.25}]-CMC_{2.5}) produced release kinetics that were in between those of dextran₆-CMC_{2.5} and CMC_{2.5}-CMC_{2.5}. At 7 h dextran₆-CMC_{2.5}, [dextran₃+ CMC_{1.25}]-CMC_{2.5} and CMC_{2.5}-CMC_{2.5} released 44, 71 and 88% of total FITC-albumin, respectively, $p < 0.001$).

When the FITC-albumin was packaged with the 2.5% CMC-ADH instead of the 6% dextran-CHO as above, burst release was higher (13.5% vs. 3.4% in the first 10 min; $p < 0.001$; Fig 3C). The long-term release kinetics were quite similar, however.

For all the hydrogels, more than 50% of total release occurred in the first 24 hr. However, release was consistently slower when dextran was incorporated. For example, at 24hr, 53% of FITC-albumin was released from dextran₆-CMC_{2.5} compared to, 94% for CMC_{2.5}-CMC_{2.5} and 97% for HA_{2.5}-CMC_{2.5} ($p < 0.001$, Fig. 3A-C). Hydrogels that did not contain dextran released more than 85% by 6 hr, (Fig. 3A). Consequently, dextran-CHO/CMC-ADH gels were used in all subsequent experiments.

3.3. Release kinetics and bioactivity of proteins released from gelatin-containing cross-linked hydrogels

Incorporation of gelatin into the hydrogels slowed release considerably. When the gelatin and FITC-albumin were packaged with the CMC-ADH, 25.6% of total FITC-albumin was released in 24 h, while 45.2% was released if packaged with the dextran-CHO (Fig. 4A).

Given these results, IL-2 and gelatin were packaged with the CMC-ADH. As with FITC-albumin, the release of IL-2 was markedly slowed by the presence of gelatin (Fig. 4B). The bioactivity of the released IL-2 was assayed by an IL-2-dependent murine T-cell-proliferation assay. The IL-2 remained > 70% bioactive after almost 4 weeks of release from dextran₆-CMC_{2.5} (Fig. 4C).

3.4. Cytotoxicity

The effect of hybrid gels on mesothelial cell and macrophage cell line viability was assessed in vitro with the MTT assay. Cells were grown in the presence of 100 μ L cylindrical dextran₆-CHO-CMC_{2.5} gels, for up to 3 weeks with media changes every 3 days. Hydrogels with or without 2% gelatin were used; albumin and IL-2 were not added since the subject of inquiry is the delivery vehicle, not the specific protein payload. For the 3 weeks tested, both mesothelial (Fig. 5A) and macrophage (Fig. 5B) cell lines showed viability greater than 85% of that of cells not exposed to any gels; the incorporation of gelatin into the hydrogels did not affect viability. There were no statistically significant differences between groups.

3.5 Biocompatibility

Mice were injected subcutaneously between the scapulae with 0.1 mL of dextran₆-CMC_{2.5} with and without gelatin. The hydrogels were still localized at the site of injection 21 days following injection. At that time, animals were euthanized and residual gelatin was harvested along with surrounding tissues. Light microscopy of hematoxylin-eosin-stained sections of the harvested site of injection (Fig. 6) revealed the presence of a mild-to-moderate inflammatory reaction (Fig. 6 all panels) consistent with reactions we have seen to other injected hydrogels, including ones that were suitable for preventing peritoneal adhesions [18]. There were some foamy macrophages (Fig. 6A) - macrophages with lucencies within their cytoplasm reflecting ingested debris. Tissue reaction was generally benign, although there were rare centralized nuclei in muscle cells (Fig. 6B), suggesting mild muscle injury in the immediate vicinity of the gels. No differences could be observed between the two groups.

4. Discussion

We have developed a system consisting of two injectable fluids that cross-link in situ that releases proteins from an in situ cross-linkable hydrogel. Such formulations would be easy to handle and - being injectable - could be delivered via minimally invasive means. They could be injected into tissue, or used to coat surfaces. Their relatively rapid gelation times would allow the operator control over what part of a surface to coat, and their in situ cross-linking would allow the coating to follow the contours of underlying tissues (unlike pre-formed polymer sheets). This combination of properties could make them particularly useful in anatomically difficult locations to coat, such as the peritoneum, the tympanic membrane, or the inside of the pericardial sac.

The system could be used as a depot for local or systemic release of protein/peptides. We were able to tune protein release by altering the initial polysaccharide composition. Furthermore, the incorporation of gelatin significantly reduced the rate of release of both proteins studied. Incorporation of gelatin did not appear to harm gel formation. In fact, it reduced the gelation time (Fig. 1) possibly because of an increase in viscosity and/or the addition of functional groups for cross linking. As can be seen from the SEM (Fig. 2), the addition of gelatin made the hydrogels less porous, which might have contributed to slowing the release profile.

These formulations provided prolonged release of proteins. In the absence of gelatin, the protein was mostly released within 4–5 days; the addition of gelatin prolonged release to more than 3 weeks. Moreover, the protein that was released maintained high activity (>70% activity for IL-2 throughout the time course).

It has previously been shown that gelatin microgels are good candidates for slow release of proteins [2, 6]. One of the problems with gelatin- and polymer-based formulations for the release of proteins is the need for additives such as surfactants and cross-linkers as well as the use of sonication, all of which can adversely affect the encapsulated protein/peptide [15]. Here we developed a system that spontaneously cross-linked by hydrazide-aldehyde bond formation with no need of chemical additive or energy source. The system can be easily tuned (e.g. polymer molecular weight, type, cross-linker density) and can allow for a broad range of peptide/protein payloads and release profiles. Precursor selection and the incorporation of gelatin can reduce the burst release and increase the duration of release. The various hydrogel combinations maintained their injectability after addition of gelatin. The hydrogels showed minimal cytotoxicity in vitro, and tissue reaction was benign. This does not exclude the possibility that high local concentrations of peptide/proteins could cause local tissue irritation, even if the base system appears to be relatively benign.

5. Conclusion

In situ cross-linking polysaccharide-based hydrogels containing gelatin allow prolonged release of proteins with retention of protein activity. These formulations obviate the need for potentially toxic reagents, and tissue reaction to them is benign.

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Figure 1.
A. Gelation times of hydrogels. B. % swelling of hydrogels. Data are means $\pm$ standard deviations ($n=5$, $*p < 0.01$, $**p < 0.001$). In all cases the gelatin was in the syringe containing the aldehyde.

Figure 2. Scanning electron micrograph of lyophilized dextran-CMC with and without 2% gelatin. Scale bars are 500 and 100 μm for the upper and lower panels, respectively.

Figure 3.

In vitro release of FITC-albumin from hydrogels in PBS at 37 °C. A. Release from various polysaccharide-CMC gels. B. Effect of combining dextran₆-CHO and CMC_{2.5}-CHO. C. Effect of packaging FITC-albumin with the -CHO or -ADH modified polysaccharide. Data are means $\pm$ standard deviations (n = 4). Statistical comparisons are discussed in the text.

Figure 4.

In vitro release of proteins from hydrogels containing 2% gelatin in PBS at 37 °C. A. Effect of packaging FITC-albumin and gelatin with the –CHO or –ADH modified polysaccharide. B. Effect of gelatin on the release of IL-2 from dextran₆-CMC_{2.5}. C. Bioactivity of eluted IL-2. Data are means with standard deviations (n = 4). Statistical comparisons are discussed in the text.

Figure 5. Viability of (A) mesothelial and (B) macrophage cell lines in the presence of hybrid gels (dextran₆-CMC_{2.5} with or without gelatin). Data are means with standard deviations (n=4).

Figure 6. Representative photomicrographs of hematoxylin-eosin stained sections of the site of injection of dextran₆-CMC_{2.5} after 21 days. A. Low-powered view showing the interface between the tissue and the remnant of the gel (left lower corner) containing foamy macrophages. B. Mild muscle injury: the arrow indicates a centralized nucleus. C. Inflammatory cells surrounding the hydrogel remnants.

Table 1

Summary of hydrogels compositions for Albumin-FITC release

Composition				
First syringe (0.5 mL)			Second syringe (0.5 mL)	
X-CHO			2.5% CMC-ADH	
	Albumin-FITC	2% Gelatin	Albumin-FITC	2% Gelatin
6% Dextran	–	–	+	–
	+	–	–	–
	+	+	–	–
	–	–	+	+
2.5% CMC	+	–	–	–
2.5% HA	+	–	–	–