

Video Article

# Synthesis and Characterization of Charged Hydrogels for the Extended Delivery of Vancomycin

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## Abstract

Drug eluting polymers are of great interest due to their utility in cancer treatment, tissue regeneration, and infection prevention. It is desirable for these materials to offer tunable structural characteristics and molecular affinities, as well as excellent biocompatibility. In the context of surgery, infection of the wound site remains a significant risk for any procedure. It is standard practice to deliver systemic antimicrobial agents to combat infection of the surgical wound. To complement systemic therapy, an implantable, local delivery vehicle would be ideal. Our group investigated the utility of oligo(poly(ethylene glycol)fumarate) / sodium methacrylate (OPF/SMA) charged copolymers as a local delivery vehicle for the antimicrobial drug vancomycin. Here, we describe the methods of OPF synthesis, and OPF/SMA hydrogel production. Additionally, we demonstrate a facile chromatographic method for quantification of vancomycin loading and release from the OPF/SMA hydrogels. OPF-based biopolymers have proven to be biocompatible *in vivo* and have wide-ranging applications due to their ability to be crosslinked chemically or via ultra-violet irradiation. OPF is readily functionalized with charged groups, or mixed with other polymers to create co-polymers with optimal structural properties or unique responses to environmental stimuli. This study utilizes an array of charged OPF/SMA co-polymers to develop a controlled release vehicle for the broad spectrum antibiotic vancomycin based upon charge-charge interactions. Hydrogel loading of vancomycin can be quantified with a simple high performance liquid chromatography method. Vancomycin release from the hydrogels is initiated in the presence of free ions in solution, and release is buffered by free charges on the pendant chains within the crosslinked hydrogel. In sum, we report an efficient method for synthesis and characterization of the OPF/SMA hydrogels, and quantification of their ability to load and release vancomycin.

## Introduction

Stimuli responsive hydrogels are of interest for their wide-ranging applications in enzyme / antibody immobilization, analytical techniques, nucleic acid and protein delivery, and cell encapsulation<sup>1-7</sup>. Many such materials are polymer networks which contain charged pendant chains - thus electric field, temperature, pH, and ionic strength modulate the physical characteristics of the hydrogel. Thus, response to changes in physical stimuli can be tailored to suit desired applications for hydrogels. Oligo(poly(ethylene glycol)fumarate) (OPF) polymers and co-polymer films or particulate formulations have been used for a number of chemical and biological applications. Their ease of use makes them attractive candidate polymers for crosslinking with other synthetic or naturally found polymer chains, or functionalization with pendant groups. OPF polymers have been shown to be useful in regenerative applications, as well as for drug delivery purposes<sup>8-10</sup>. Previously, our group reported the synthesis of positively and negatively charged OPF hydrogels<sup>11,12</sup>. This led us to consider the possibility of exploiting an affinity based interaction between the polymer and a charged drug for an easily produced, bioresorbable drug delivery device.

Vancomycin, is a broadly active, positively charged, glycopeptide antimicrobial drug which was first isolated from *Amycolatopsis orientalis*. Vancomycin efficacy is limited by a number of factors: systemic delivery often results in an immunologic adverse event termed "red man syndrome". Additionally, serum concentrations of vancomycin correlate poorly with tissue exposure - and clinical outcome<sup>13-15</sup>. This unpredictability has steered some clinicians away from vancomycin use when other antibiotics may be chosen. Despite such troubles, vancomycin use has gradually increased recently<sup>16</sup>. In an attempt to avoid dose-limiting systemic side effects, a number of surgical teams have reported the use of powdered vancomycin hydrochloride as an intra-operative wound dressing for prevention of bacterial colonization of the wound site<sup>17,18</sup>. This means of delivering concentrated doses of vancomycin to the wound site has been demonstrated to be especially effective in spine surgery<sup>19</sup>. Unfortunately, the high aqueous solubility, poor absorption, and quick elimination of vancomycin reduces its ability to distribute throughout local tissue, and limits its efficacy when administered topically. Thus, local delivery via a bioresorbable, implantable material would improve local tissue exposure to vancomycin. Environmentally responsive biopolymers such as OPF would provide an ideal solution to control

dosing of antibiotics for such applications. This study focuses on the use of OPF due to its previously characterized biocompatibility in cellular and animal models, and facile synthesis and customization.

The wide-spectrum of applications for OPF, and derived co-polymers, highlight the utility of efficient methods for polymer and hydrogel synthesis and purification. Vancomycin, as well as other charged molecules, may be loaded onto hydrogel matrices containing pendant charges through affinity based interactions. Additional functionalization of such hydrogels is possible through incorporation of micro- or nanospheres loaded with bioactive molecules, or by producing a porous hydrogel matrix through various means, such as salt leaching. Thus, fully customizable hydrogel matrices for a large spectrum of applications are achievable from the same starting hydrogel material, the synthesis of which we describe here, and have utilized previously<sup>20</sup>. After drug loading, the OPF/SMA co-polymer depends on an ion-exchange mechanism in the presence of physiologic salt concentrations to release the drug. In this study, we demonstrate an efficient method to synthesize an array of OPF/SMA charged co-polymers for use as implantable, drug eluting devices. Additionally, we demonstrate a simple, reverse-phase high performance liquid chromatography (HPLC)-based method for detection and quantification of vancomycin.

## Protocol

### 1. OPF/SMA hydrogel synthesis

1. Prepare the OPF macromer beginning with 10,000 Da poly(ethylene glycol) according to methods described by Kinard, *et al.*<sup>21</sup>.
2. Create a 10% w/v solution of photoinitiator by mixing with double-distilled water (ddH<sub>2</sub>O). For example, add 100 mg of photoinitiator to 10 mL of ddH<sub>2</sub>O. Add low heat (55 °C), and gently stir to dissolve completely.
3. In a 50 mL conical tube, mix the desired amount of 10,000 Da molecular weight OPF macromer with 950 µL of ddH<sub>2</sub>O and 750 µL of the photoinitiator solution. The proper amount of OPF depends on the desired ratio of OPF/SMA. For example, create a 10% SMA co-polymer by beginning with 900 mg of OPF, and adding 100 mg of SMA in step 1.4.
  1. For each desired OPF/SMA copolymer, prepare a separate 50 mL conical tube with the reaction mix. Stir the OPF, water, and photoinitiator solution in each conical tube vigorously with a spatula. Incubate at 37 °C for 10 min in a water bath.
4. Remove the conical tube containing the reaction mix from the water bath and add 300 µL of 1-vinyl-2-pyrrolidinone (NVP) by pipette, and add the desired amount of sodium methacrylate (SMA) powder. Mix with a clean metal spatula until the SMA is dissolved completely.
5. Centrifuge the 50 mL conical tube containing the reaction mix for 5 min at 3,000 x g to remove bubbles from the solution. The reaction mix is now ready to be molded and photo-crosslinked.
6. Pour the reaction mix into 1"x1" square, 1/32" thick PTFE molds covered by 1/8" thick soda lime window glass plates and seal with metal binder clips (two for each mold). Pour approximately 500 µL of reaction mix into each mold, or until the reaction mix has filled the boundaries of the mold. Typically, use one mold for each OPF/SMA copolymer film being produced.
7. Place the prepared molds into a room temperature UV oven for 30 min to crosslink the polymer hydrogel.
8. Once the crosslinking has finished, remove the samples from the oven, open the molds and cut the hydrogel film into 3 mm discs using a 3 mm diameter biopsy punch. The molds may be difficult to open as the hydrogel has crosslinked within it. A metal spatula may be used to help open the molds. More than thirty 3 mm discs may be cut from a single prepared hydrogel film.
9. Place the freshly cut hydrogel discs into 50 kDa exclusion standard dialysis tubing, separating each copolymer group into unique dialysis bags and labelling carefully. Allow the discs to dialyze in 1 L of ddH<sub>2</sub>O for 72 h in order to remove any uncrosslinked, soluble material.
10. Using forceps, remove the dialyzed hydrogels from the tubing and allow to dry for 2 h at room temperature. Typically, place the hydrogels on 10 cm dishes covered with aluminum foil during the drying process. Place the hydrogels in a desiccating oven for 8 h (or overnight) in order to remove excess water from the samples. The clean hydrogels are now ready for use.

### 2. Hydrogel characterization

1. **OPF/SMA hydrogel swelling ratio calculation**
  1. Weigh the dried OPF/SMA hydrogels. It was typical to find that a dried 3 mm hydrogel disc weighed between 4.0 and 6.0 mg. Place each hydrogel to be used for swelling ratio calculations in a 50 mL conical tube containing a solution of ddH<sub>2</sub>O, Dulbecco's phosphate buffered saline (DPBS), or 400 µg/mL vancomycin in ddH<sub>2</sub>O.
  2. Allow hydrogels to incubate in the solution at room temperature for 24 h.
  3. Remove hydrogels from the solution with forceps and dry briefly to remove excess water. Measure the weight of the swollen hydrogels.
  4. Calculate the swelling ratio of the hydrogels by subtracting the dried mass (Md) from the swollen mass (Ms) and dividing by the dried mass, such that the swelling ratio = (Ms-Md) / Md.
2. **Perform thermal gravimetric analysis of OPF/SMA hydrogels**
  1. Load dialyzed and dried hydrogel discs onto the sample plate of a thermal gravimetric analyzer.
  2. Heat the samples at a ramp rate of 20 °C/min from 50 °C to 1000 °C under an inert atmosphere.

NOTE: Increased degradation near 140 °C is observed in the samples containing higher mass percentage of SMA.

### 3. Hydrogel loading with vancomycin

1. Prepare a fresh, stock solution of concentrated vancomycin in water (e.g. 10 mg/mL) by weighing powdered vancomycin into a low affinity microcentrifuge tube and adding an appropriate volume of ddH<sub>2</sub>O. Use this solution to create the loading solutions by standard dilution. Be sure to mix well by vortexing before use.
2. Add 1 mL of working concentration (e.g. 400 µg/mL) of vancomycin solution to a 2 mL, low-affinity microcentrifuge tube. Keep a stock of this solution for further use as a quantification standard. Prepare enough vancomycin solution, hydrogels, and tubes for all groups and replicates.

3. Weigh clean, dried OPF/SMA hydrogels, and place individual hydrogels into the microcentrifuge tubes containing the working concentration vancomycin solution. Mix well and allow to incubate up to 72 h to allow vancomycin to load onto the hydrogel scaffold. The hydrogels will also swell during this period.
4. After loading, remove the hydrogels from the microcentrifuge tubes with a forceps and allow to dry at room temperature for 2 h by placing on 10 cm dishes covered with aluminum foil. Retain the loading solutions for determination of vancomycin concentration in solution, and calculation of loading efficiency.
5. Place the drug loaded hydrogels in a desiccating oven for 8 h in order to completely remove excess liquid from the hydrogels.
6. Measure initial and final concentrations of loading solutions (see step 5 for quantification method) to determine the mass of vancomycin loaded per scaffold. Calculate loading efficiency by determining the unit mass of vancomycin achieved per unit mass of hydrogel (e.g.  $\mu\text{g}$  VCM/mg hydrogel).

#### 4. Vancomycin release from OPF/SMA hydrogels

1. Place the loaded and dried OPF/SMA hydrogels into fresh 2 mL, low-affinity microcentrifuge tubes containing 1 mL of DPBS. At this time, Step 5.1 may be begun as well. Keep the vancomycin solutions to be used as standards at 37 °C for the entire release period in order to control for degradation of the drug over time.
2. Incubate the hydrogels in DPBS at 37 °C for the desired release period.
3. Following drug elution, remove samples from the tubes, retaining the elution solution. Keep the solution containing eluted drug for analysis via HPLC.

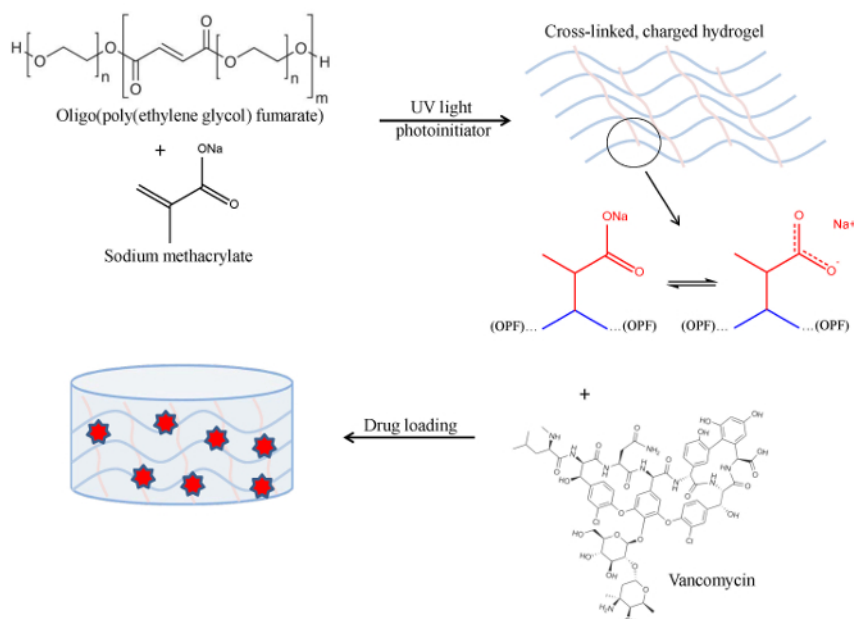
#### 5. High performance liquid chromatography quantification of solution vancomycin

1. Prepare a standard curve of vancomycin from stock solution used to prepare the vancomycin loading solution (as in Step 3.1, except diluted with DPBS). A recommended standard curve may include 8 standard concentrations ranging from 1  $\mu\text{g/mL}$  to 1000  $\mu\text{g/mL}$ .
2. Equilibrate a reverse-phase  $\text{C}_{18}$  HPLC column (150 x 4.6 mm i.d., 2.1  $\mu\text{m}$  particle size) with pH 5.0 dibasic potassium phosphate buffer (5 mM, pH = 2.8) and acetonitrile at a ratio of 80/20, respectively. Equilibrate the column for at least 10 min prior to any samples being run.
3. Set the mobile phase flow rate to 0.5 mL/min, and prepare ultra-violet detection of eluents at 282 nm.
4. Load the standard samples of vancomycin in water onto the HPLC (for drug release solutions, prepare standards in DPBS). Under the described conditions, vancomycin elution time is approximately 4.9 min.
5. Run all samples through the HPLC, ordering the samples such that solutions expected to contain less vancomycin are run first. This prevents contamination from higher concentration samples in the event that all vancomycin does not elute or rinse properly. Calculate signal peak area at 282 nm wavelength detection. Allow for 10 min per sample in order to remove any excess drug or polymer degradation materials, and to re-equilibrate the column prior to the next sample.

### Representative Results

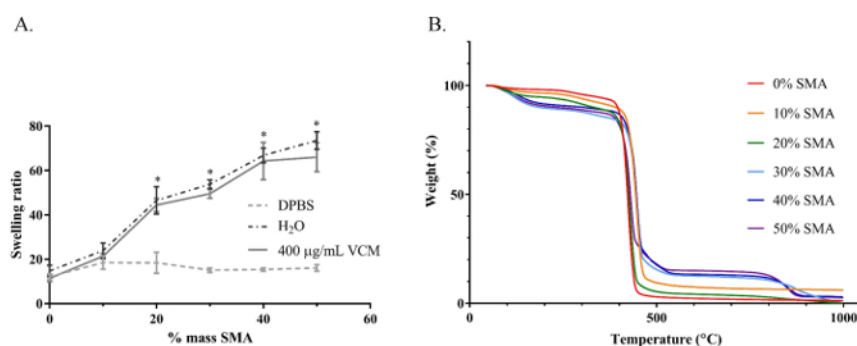
This method describes the synthesis and characterization of OPF/SMA hydrogels, and the quantification of their vancomycin loading and release properties. In **Figure 1**, the general structure of the hydrogels is depicted with their idealized loading of vancomycin. OPF macromer chains are crosslinked with SMA, resulting in free negative charges within the hydrogel co-polymer network. **Figure 2** establishes the change in hydrogel physical properties due to charge incorporation from SMA crosslinking. Swelling ratio changes (**2A**) due to SMA weight percent, and additional TGA mass losses (**2B**) indicate the structural differences.

Representative charts from the HPLC method are shown in **Figure 3**, proving the efficiency and sensitivity of the quantitation method. The area under the vancomycin signal was calculated for each sample and compared to a linear standard curve of vancomycin. Various loading experiments are shown in **Figure 4**, showing the time (**4A**) and charge (**4B**) dependent nature of the interaction. Maximal loading was achieved at 24 h, and hydrogels loading efficiency increased consistently even with high concentrations of vancomycin which were well past physiologically relevant concentrations (**4C**). The loading procedure is diagrammed in **Figure 4D**. Finally, **Figure 5A** depicts the drug release period. **Figure 5B** demonstrates the ability of hydrogel charge to delay the release of vancomycin over time by comparing the uncharged OPF, and high charge (50% SMA) OPF polymers. All release curves from the OPF/SMA polymers are plotted in **Figure 5C**, showing the steady decrease in release rate with increased charge.



**Figure 1: Schematic depicting synthesis of OPF/SMA hydrogels.**

OPF/SMA hydrogels synthesized as described. Idealized OPF chain represented in blue, SMA is depicted in red, crosslinked between OPF chains. Vancomycin shown as red stars within the hydrogel. This figure has been modified from Gustafson CT, *et al*<sup>20</sup>. [Please click here to view a larger version of this figure.](#)



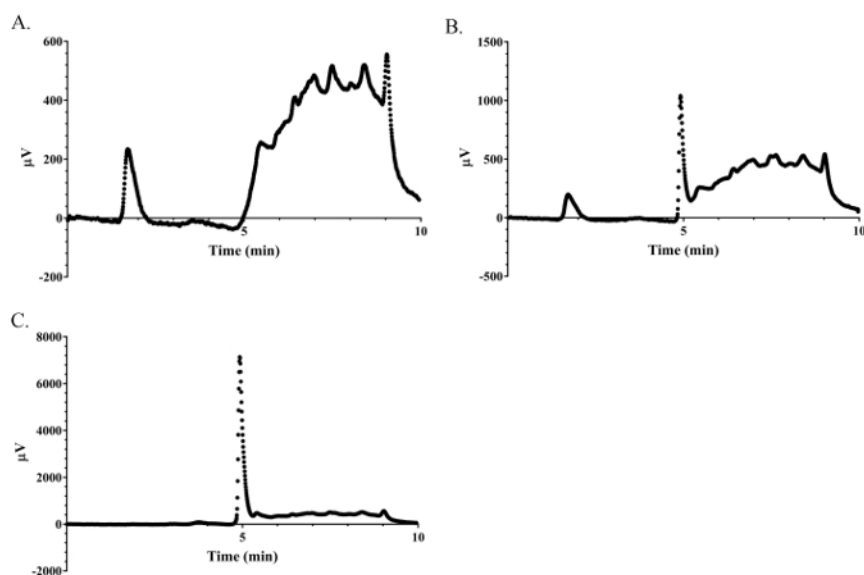
**Figure 2: Physical characterization of OPF/SMA hydrogel discs.**

A) Hydrogel discs (as described) were swollen in either ddH<sub>2</sub>O, DPBS, or a solution of vancomycin hydrochloride (400 µg/mL) in ddH<sub>2</sub>O. The hydrogels were dried, weighed, then swollen again and weighed again. Swelling ratio calculated as described in Protocol section 2.1. Error bars represent  $\pm$  one standard deviation, N = 3 in all groups. (\*) indicates a statistically significant difference ( $p < 0.05$ ) between DPBS and ddH<sub>2</sub>O groups. B) Thermogravimetric analysis of OPF/SMA hydrogel films. Three significant degradations could be observed in some groups, while one degradation (T2) was consistent throughout all formulations. This figure has been modified from Gustafson CT, *et al*<sup>20</sup>. [Please click here to view a larger version of this figure.](#)

	OPF	OPF/SMA10	OPF/SMA20	OPF/SMA30	OPF/SMA40	OPF/SMA50
T1 Onset temp (°C)	N/A	63.622	68.626	66.109	68.14	89.414
T1 End temp (°C)	N/A	72.415	131.456	141.931	143.296	155.99
T1 % change	N/A	4.187	5.471	10.715	8.592	8.986
T2 Onset temp (°C)	405.319	410.194	410	412.431	412.413	411.852
T2 End temp (°C)	438.71	444.254	443.431	444.095	444.268	444.793
T <sub>d</sub> (°C)	422.944	429.878	428.233	427.98	431.411	429.29
T2 % change	96.443	88.582	90.3524	77.143	76.852	73.491
T3 Onset temp (°C)	N/A	N/A	N/A	812.214	799.449	809.768
T3 End temp (°C)	N/A	N/A	N/A	891.878	861.605	871.02
T3 % change	N/A	N/A	N/A	11.728	9.632	11.803
Final % remaining	1.106	5.883	0.025	0.534	2.687	2.574

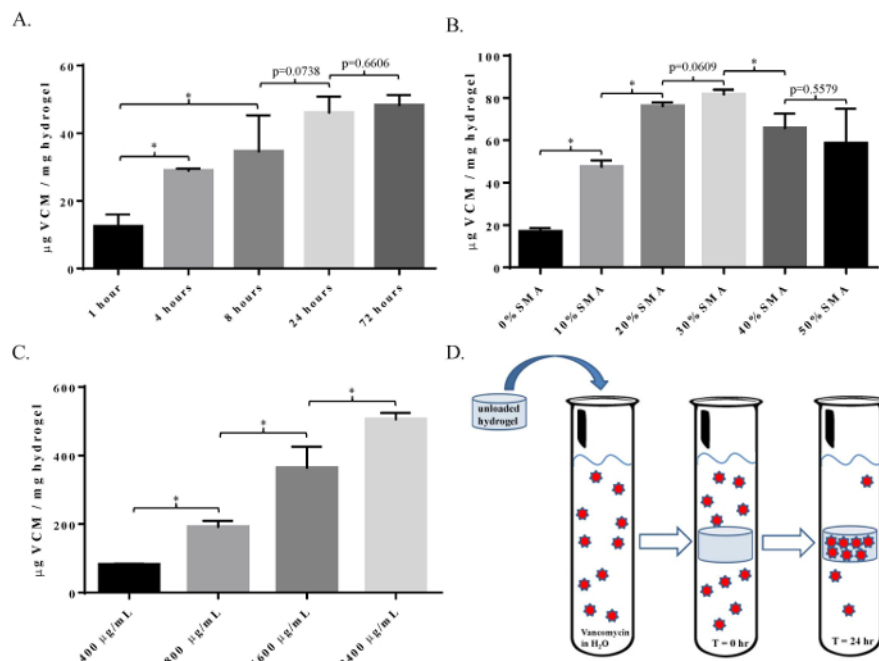
**Table 1: Thermal properties of OPF/SMA hydrogels.**

Analysis of TGA scans and compilation of average degradation temperatures. Values are a mean calculated from replicate experiments (N=2). Undetectable value denoted by (N/A). This figure has been modified from Gustafson CT, *et al*<sup>20</sup>.



**Figure 3: Representative HPLC results.**

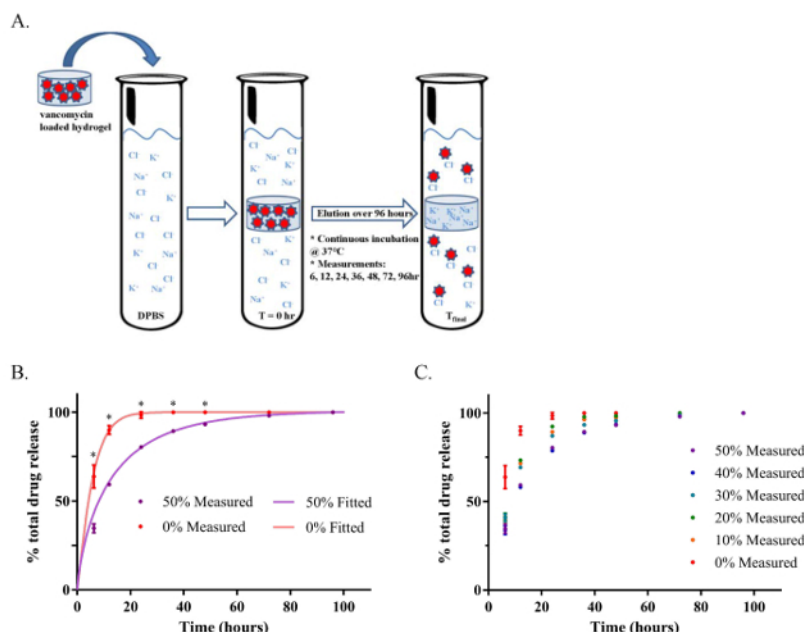
A) Blank sample used in the standard curve for the 24-hour release time point. B) Vancomycin standard at 10 μg/mL used in the standard curve for the 24-hour time point. Retention time = 4.925 min, concentration of the sample = 9.685 μg/mL (back-calculated based off the standard curve). C) Sample 50B from a drug loading experiment, drug concentration determined from a set of drug standards handled and ran in parallel to the sample solutions. This figure has been modified from Gustafson CT, *et al*<sup>20</sup>. [Please click here to view a larger version of this figure.](#)



**Figure 4: Analysis of vancomycin loading in OPF/SMA hydrogels.**

A) OPF/SMA 40% hydrogels were incubated with vancomycin at 400 µg/mL for 1 hour, 4 h, 8 h, 24 h, or 72 h. The concentrations of vancomycin in solution were then measured with HPLC coupled to UV-Vis detection at 282 nm and drug loading was determined by subtracting the initial concentration of drug from the final concentration after completion of vancomycin loading. B) Hydrogels were incubated for 24 h, samples were collected, and the concentration of vancomycin in solution was determined as described. Amount of drug loaded was calculated and loading efficiency was determined by dividing the mass of loaded drug by the dried hydrogel mass, such that loading efficiency is represented as µg vancomycin per mg hydrogel. C) Maximal drug loading was determined by incubating OPF/SMA 30% hydrogel samples in increasingly concentrated solutions of vancomycin in double distilled water for 24 h. Loading efficiency ( $Eff_i$ ) was determined by measurement of the final drug concentration in the distilled water ( $C_f$ ) after completion of the loading cycle, followed by comparison to concentration of initial solution ( $C_i$ ) via the equation ( $Eff_i$ ) =  $\{[(C_f) - (C_i)] / (C_i)\} \times 100$  %. D) Diagram representing drug loading experiment. In all experiments, concentrations of vancomycin remaining were determined from a standard curve of vancomycin solution that was incubated under identical conditions to the sample of interest. Error bars represent  $\pm$  one standard deviation, N=3 in all groups. Statistical representation: (\*) indicates  $p < 0.05$ . This figure has been modified from Gustafson CT, *et al*<sup>20</sup>. [Please click here to view a larger version of this figure.](#)





**Figure 5: Vancomycin release from OPF/SMA hydrogels.**

A) Diagram representing release of vancomycin from charged hydrogels. OPF/SMA hydrogels loaded with vancomycin were placed in DPBS solutions and incubated at 37 °C for 96 h. Solutions were collected and fresh DPBS was replaced at the indicated time points. The concentration of eluted vancomycin was measured by HPLC. B) Cumulative release is represented as a percentage of the total amount of measured drug released. Error bars represent  $\pm$  one standard deviation,  $N = 3$  in all groups. Statistical representation: (\*) indicates  $p < 0.05$ . The fitting function for the cumulative release is given by:  $R(t) = M[1 - \exp(-(t/\tau)^b)]$ , where  $M$  is maximally achieved cumulative release (100%) and  $\tau$  is the time when approximately 63.2% corresponding to  $M[1 - \exp(-1)]$  is achieved. Parameter  $b$  determines the stretching of the exponential function. The smaller its value, the slower is the convergence to 100%. C) All measured values for each experimental group plotted together as a function of time. Percent of the total drug release is plotted on the x-axis. This figure has been modified from Gustafson CT, *et al*<sup>20</sup>. [Please click here to view a larger version of this figure.](#)

## Discussion

The methods described here outline a facile method to synthesize OPF/SMA charged hydrogels, and to quantify the elution of a drug (vancomycin) from the hydrogel matrix. While the protocol is relatively straightforward, it may be useful to note several pitfalls which should be avoided during the synthesis, loading, and release in order to achieve more accurate results. During the hydrogel synthesis (see step 1.6), it is important to avoid the incorporation of air pockets in the co-polymer while pouring into a mold. This can be avoided by proper centrifugation (step 1.5) and by ensuring that no air becomes trapped in the mold before cross-linking. We also found that, if desiccated too rapidly, the hydrogels which contained higher amounts of SMA were more likely to become brittle and degrade quickly within the drying oven (step 1.10). To avoid this problem, it was important to briefly dry the cross-linked and dialyzed hydrogels at room temperature and pressure for at least two h prior to placing in the desiccator. We found that longer drying periods (e.g. overnight) at room temperature and pressure, prior to desiccation, was also acceptable. This pre-desiccation step allowed some water to escape the hydrogel, and resulted in more efficient production of viable, high charge hydrogels. While quantifying vancomycin concentrations (step 5), we found that allowing a total run time of 10 min allowed for accurate quantification of vancomycin. Although a shorter run time may be utilized (e.g. by using a higher flow rate), we noted that this may decrease the accuracy of the measurements. Additionally, we recommend sequencing the order of the samples to be run on the HPLC, such that those known to contain less vancomycin would be run first. In this way, if a drug sample does not completely elute from the column in the previous run, it is less likely to significantly alter the results of the next sample.

This method may be modified for the inclusion of additional materials within the hydrogel, such as salt crystals, in order to provide a porous matrix. Additionally, the hydrogel may be functionalized with microparticles to entrap additional compounds which may not be compatible with the hydrogel loading mechanism (e.g. highly lipophilic molecules). The ability to modify and functionalize the OPF polymer and co-polymer hydrogels is one of their greatest strengths. Our goal is for this method to provide a base from which further, more sophisticated polymeric systems may be constructed. One of the limitations of the OPF/SMA hydrogels is their dependence on charge interactions for drug loading, and the hydrophilic nature of the loading mechanism. Both properties exclude the use of lipophilic molecules for direct loading and release, thus calling for further modification of the hydrogel in order to facilitate delivery of hydrophobic compounds. Indeed, liposomal formulations for many bioactive molecules are commonplace, and it can be easily postulated that similar encapsulating delivery systems could be incorporated into the hydrogel matrix.

The accurate detection of vancomycin in solution was essential to obtain quantifiable loading and release results for this report. The HPLC method reported here could, with slight modification, be converted to detect serum concentrations of vancomycin as well. We hope that this report will aid further development of efficient means to detect vancomycin in tissue and blood samples. As with any other compound, degradation of the material while in solution was a significant concern. Thus, the vancomycin loading and release samples were run as quickly as possible following use, and were matched with standard curves which had been prepared and handled in parallel. We found that storing

drug samples at -20 °C, and thawing to measure at a later time resulted in significant inaccuracies, and we highly recommend measurements to be taken as quickly as possible following sample collection. A similar drug loading and release protocol may be followed with the use of other compounds which may have variable degradation properties in aqueous solution. We recommend optimization of material handling and storage, loading kinetics, and affinity for various chromatography columns in order to fine-tune methods by which to quantify loading and release kinetics.

Additional polymeric materials exist which may be formed into hydrogels in a similar fashion<sup>22</sup>. Natural and synthetic compounds such as chitosan, hydroxyethyl methacrylate, silicone, or collagen are among a number of starting materials favorable for synthesis into hydrogel formulations<sup>23-26</sup>. Structural requirements of the hydrogel application, the desired degradative timeline, biocompatibility, as well as the nature of additional incorporated elements such as nanoparticles, will determine the optimal material for use. The OPF/SMA hydrogel co-polymers reported here are a flexible and customizable material with a wide range of uses and applications. Additionally, excellent biocompatibility has been observed with the use of various OPF polymers, including the OPF/SMA co-polymer. This report details the necessary steps to synthesize a range of OPF/SMA co-polymer hydrogels, and provides a protocol for the quantitation of loading and release of the glycopeptide drug vancomycin onto the hydrogels.

## Disclosures

The authors of this manuscript report the following competing interests: patent for "Photocrosslinkable oligo(poly (ethylene glycol) fumarate) hydrogels for cell and drug delivery." Inventors: Mahrokh Dadsetan, Michael Yaszemski, Lichun Lu. Filing Date: March 23, 2006. Patent number: US 8343527 B2. This competing interest affects co-authors MJY and MD. It does not alter the authors' adherence to JoVE policies on sharing data and materials.

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