Photonic Crystal Kinase Biosensor

Kelsey I. MacConaghy, Christopher I. Geary, Joel L. Kaar*, Mark P. Stoykovich*

Department of Chemical and Biological Engineering, University of Colorado, Boulder, Colorado 80309, United States

* Corresponding Author
joel.kaar@colorado.edu; mark.stoykovich@colorado.edu

Electronic Supporting Information (ESI)
Detailed materials and methods, theoretical model of hydrogel-encapsulated CCA swelling, and results on the biosensor response as a function of known extents of phosphorylation and phosphatase activity. This material is available free of charge via the Internet at http://pubs.acs.org.

I. Materials and methods

Peptide synthesis and purification
LRRASLG was synthesized by standard solid-phase peptide synthesis methods. Fluorenlymethoxycarbonyl (Fmoc)-protected amino acids, MBHA Rink amide resin, and (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylyuronium hexafluorophosphate (HBTU) were purchased from Chem-Impex International, Inc. Solvents (ACS grade) were purchased from Fisher Scientific and used as received without further purification. Manual solid-phase synthesis was performed under constant agitation at room temperature. Prior to each amino acid coupling step, a solution of 20 vol% piperidine in DMF was used for Fmoc deprotection of the N-terminal amine of the peptide. The amino acid to be coupled was pre-activated with HBTU and N,N-diisopropylethylamine (DIPEA) in DMF (4 mol eq. amino acid : 3.96 mol eq. HBTU : 6 mol eq. DIPEA) before being added to the reaction vessel. After addition of the final amino acid, the final N-terminus Fmoc group was removed and the peptide was cleaved from the resin through agitation in a solution of trifluoroacetic acid (TFA), water, and triisopropylsilane (95 vol eq.: 2.5 vol eq.: 2.5 vol eq.). The peptide was recovered by precipitation in ethyl ether and dried. Peptide was subsequently resuspended in water and 0.1 vol% TFA to a final concentration of 2 mg/mL and filtered prior to HPLC purification. The product was purified by reverse-phase HPLC (Agilent 1100) using a Phenomenex Jupiter column (stationary phase: 10 µm, dC12) and linear gradient of 0% to 95% acetonitrile in water (HPLC grade), both with 0.1 vol% TFA. After purification, the peptide product was rotary evaporated to remove excess solvents and lyophilized. The mass and purity of the LRRASLG product were verified using matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF, ABI Voyager-DE STR) at the University of Colorado Mass Spectrometry Central Analytical Lab. For control experiments using phosphorylated peptide, crude LRRApSLG was purchased from GenScript, Inc. and purified by HPLC following the same procedure as for non-phosphorylated LRRASLG.

Synthesis of colloidal suspensions of PS spheres
Monodisperse, negatively-charged polystyrene (PS) spheres were synthesized by emulsion polymerization as described elsewhere. The colloidal suspension of PS spheres utilized for the presented experiments contained 110 nm diameter PS particles with a polydispersity of ~4% at a concentration of ~11 wt% in water (Fig. S1). Particles were stored with BioRad mixed bed resin (AG 501-X8) to remove ion impurities from the synthesis and to stabilize the colloidal suspension. The particle diameter and polydispersity were determined by dynamic light scattering measurements (Titan DynaPro with the Dyna V6.3.4 software package). Zeta potential was measured using a Malvern Zetasizer Nano ZS.
**Polymerization of hydrogel**

Hydrogels were synthesized by free radical polymerization using Irgacure 2959 (BASF) as the photoinitiator. Solutions containing 0.1 g acrylamide monomer, 2.5 mg N,N’-methylenebisacrylamide, and 1.5 g of the suspension of colloidal PS spheres were shaken with 0.1 g of ion exchange resin (BioRad AG 501-X8). The solution was centrifuged to remove resin and photoinitiator (at 10 wt% in DMSO) was added to an overall final concentration of 0.05 wt%. The solution was then injected into a cell composed of a vinyl support film (BioRad) and 2 clean microscope slides separated by a 126.4±0.7 µm parafilm spacer. Photopolymerization was performed by flood exposure of the sample to 365 nm light from a UV mercury lamp at an irradiance of 15 mW/cm² for 1 h. Films were rinsed and equilibrated with ultrapure water.

**Peptide functionalization of hydrogel**

The polyacrylamide hydrogel backbone was hydrolyzed for 4 h at room temperature using a solution of 10 vol% N,N,N’,N’-tetramethylethylenediamine (TEMED) in a 0.1 M aqueous solution of NaOH. After extensive rinsing, the hydrogel was submerged in a 0.1 M MES buffer at pH 5 containing 0.1 M NaCl, 30 mM 1-ethyl-3-[d-dimethylaminopropyl] carbodiimide hydrochloride (EDC), and 50 mM N-hydroxysulfosuccinimide (Sulfo-NHS) for 20 min at 4 °C. The solution was then exchanged for a 0.1 M sodium phosphate buffer at pH 7.5 with 0.1 M NaCl and 100 mM LRRASLG or LRRAPSLG, and allowed to react for 16 h at room temperature. Peptide loading in the hydrogel was increased by repeating the EDC/NHS and peptide treatments for a total of 4 reaction cycles. Functionalized samples were thoroughly rinsed with and stored in sodium phosphate buffer at 4 °C.

**Kinase treatment**

The hydrogel-encapsulated crystalline colloidal arrays were immersed in 50 mM tris-HCl buffer with 10 mM MgCl₂ and 1 mM ATP. The catalytic subunit of protein kinase A (New England Biolabs) was added in varying concentrations (0 – 25 U/µL) and incubated at 30 °C for varying times (0 – 8 h). The phosphorylation reaction was stopped by inactivating the kinase via submerging the samples in water at 65 °C for 20 min. For experiments in the presence of inhibitor, PKA was pre-incubated for 10 min at 30 °C in 50 mM tris-HCl buffer with 10 mM MgCl₂, 100 µM ATP, and varying concentrations of H-89 inhibitor (Cell Signaling Technologies, 0.1 nM– 100 µM). The CCA biosensors were incubated in the enzyme-inhibitor solution for 3 h at 30 °C and then the kinase was inactivated as described above.

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**Figure S1.** Dynamic light scattering data of PS particles with 110 nm diameter and a polydispersity of ~4%.
**Phosphatase treatment**
The hydrogel-encapsulated crystalline colloidal arrays were immersed in 50 mM bis-tris-propane HCl, at pH 6.0, with 1 mM MgCl$_2$ and 0.1 mM ZnCl$_2$. Antarctic phosphatase (New England Biolabs) was added at 0.1 U/µL and incubated at 37 °C for 16 h. The dephosphorylation reaction was stopped by inactivating the phosphatase via submerging the samples in water at 70 °C for 5 min.

**Characterization of immobilized charge by hydrogel staining**
Hydrogel staining was performed using toluidine blue O (Sigma-Aldrich) to stain for negative charges and acid orange 7 (Sigma-Aldrich) to stain for positive charges. Samples were stained with toluidine blue O by rinsing thoroughly with a 0.1 mM NaOH solution and incubating with 0.5 mM stain in 0.1 mM NaOH for 3 h at 25 °C. Samples were rinsed three times with the 0.1 mM NaOH solution and the dye adsorbed to the hydrogel was extracted by incubating at room temperature with a 50 vol% aqueous acetic acid solution for 15 min. Absorbance measurements were taken at 633 nm and the dye concentration in the extracted solution was calculated using an absorptivity of $\varepsilon = 7 \times 10^4$ cm$^{-1}$ M$^{-1}$. Samples were stained with acid orange 7 by rinsing thoroughly with a 1 mM HCl solution and incubating with 0.5 mM stain in 1 mM HCl for 3 h at 25 °C. Samples were rinsed three times with the 1 mM HCl solution and dye adsorbed into the hydrogel was extracted by incubating at room temperature with a 30 vol% aqueous ethanolamine solution for 20 min. Absorbance measurements were taken at 468 nm and the dye concentration in the extracted solution was calculated using an absorptivity of $\varepsilon = 1.6 \times 10^4$ cm$^{-1}$ M$^{-1}$.

**Diffraction measurements**
Optical diffraction from the hydrogel-encapsulated CCA was measured with an Ocean Optics USB-4000 fiber-optic spectrophotometer operating in reflectance mode (angle of incidence of 15° from the sample surface normal). Spectra were collected across the UV-visible range (375 - 850 nm). Samples were thoroughly rinsed with ultrapure water to remove all mobile ions and to achieve an equilibrium extent of hydrogel swelling before each optical measurement was performed.

II. **Theoretical model of swelling in hydrogel-encapsulated CCA biosensors**
To fully understand the effect of phosphorylation and, therefore, kinase activity on the optical response of the hydrogel-encapsulated CCA biosensor, it is necessary to investigate the parameters that affect hydrogel swelling. Towards this end, a theoretical model of the hydrogel-encapsulated CCA swelling due to immobilized charge was developed to elucidate the dependency of that swelling on (i) the material properties of the hydrogel, (ii) the charge distribution immobilized in the hydrogel, and (iii) the ionic character of the surrounding environment. The developed model is based on Flory’s description of swelling in ionic polymer networks which specifies that the total osmotic pressure, at equilibrium, is equal to zero:

$$\Pi_T = \Pi_{\text{ion}} + \Pi_{\text{M}} + \Pi_{\text{E}} = 0$$  \hspace{1cm} (1)

Three contributions to the osmotic pressure are considered, including $\Pi_{\text{ion}}$, which is the osmotic pressure due to the Donnan potential that arises from the gradient in mobile ion concentration inside the hydrogel and that of the surrounding solution:

$$\Pi_{\text{ion}} = RT \Sigma (c_x - c_x^\circ)$$  \hspace{1cm} (2)

where $R$ is the universal gas constant, $T$ is the temperature, $c_x$ is the concentration of mobile ions of type $x$ inside the hydrogel, and $c_x^\circ$ is the concentration of ions in solution. $\Pi_{\text{M}}$ is the osmotic pressure associated with the free energy of mixing:

$$\Pi_{\text{M}} = -\frac{RT}{V_s} \left[ \ln \left( 1 - \frac{V_2}{V} \right) + \frac{V_2}{V} + \chi \left( \frac{V_2}{V} \right)^2 \right]$$  \hspace{1cm} (3)
where \( V_s \) is the molar solvent volume, \( V_o \) is the dry hydrogel volume, \( V \) is the current hydrogel volume, and \( \chi \) is the Flory-Huggins interaction parameter between the polymer and the solvent. Finally, \( \Pi_E \) is the osmotic pressure due to changes in the free energy upon elastic deformation of the hydrogel thin film (as confined to swelling in 1D):

\[
\Pi_E = -\frac{E}{2} \left( \frac{V_m}{V} \right)
\]

where \( E \) is the Young’s modulus and \( V_m \) is the volume of the unstrained hydrogel.

The Young’s modulus was experimentally measured by rheometry (\( E = 6 \) kPa) and the Flory-Huggins parameter was estimated from the equilibrium swelling of the hydrogel (\( \chi = 0.545 \)). The concentration of charge immobilized in the hydrogel and the corresponding mobile counterions were calculated from the staining data in Fig. 2. However, since there are both positive and negative charges immobilized in the LRRASLG-functionalized hydrogel, a portion of these charges are involved in ionic crosslinks (a concentration \( x \)) and are assumed to be inaccessible for staining. A mole balance around the concentration of positive and negative charges in the hydrogel enables calculation of the concentration of peptide immobilized in the gel (\( p \)) and unreacted carboxylate functionalities:

\[
\text{Mole balance on negative charges:} \quad C_{\text{carboxylate in hydrolysis treated hydrogel}} = C_{\text{stained unreacted carboxylate in LRRASLG treated hydrogel}} + p + x \quad (5)
\]

\[
\text{Mole balance on positive charges:} \quad 0 = 2p - C_{\text{stained arginine residues in LRRASLG treated hydrogel}} - x \quad (6)
\]

where \( C_{\text{carboxylate in hydrolysis treated hydrogel}} = 35.4 \) mM, \( C_{\text{stained unreacted carboxylate in LRRASLG treated hydrogel}} = 10.1 \) mM, and \( C_{\text{stained arginine residues in LRRASLG treated hydrogel}} = 3.3 \) mM from the staining data in Fig. 2. Solving this pair of simultaneous linear equations for \( p \) and \( x \) results in calculated concentrations of immobilized peptide of 9.5 mM and ionic crosslinks of 15.8 mM.

### III. Supplementary Results and Discussion

**Red shift in peak diffraction as a function of increasing phosphorylated peptide concentration**

The red shift in peak diffraction as a function of known phosphorylated peptide concentrations (Fig. S2) was characterized to determine the potential range of the biosensor response and to provide a standard curve for the relationship between optical response and extent of reaction. Each hydrogel was functionalized with identical total concentrations of peptide using a 100 mM peptide solution and following the procedure discussed previously. The extent of phosphorylation was controlled, however, through the molar ratio of phosphorylated and non-phosphorylated peptide added (e.g., 15 mol% extent of phosphorylation was achieved with 15 mM LRRAPSLG and 85 mM LRRASLG). Results shown in Fig. 3a indicate that the maximum red shift in peak diffraction upon kinase activity was ~80 nm. According to data presented in Fig. S2, this corresponds to an extent of phosphorylation of 26 mol%.
Figure S2. Red shift in peak diffraction as a function of extent of phosphorylation (mol%) of peptide immobilized in the hydrogel. Error bars represent ±1σ from the mean over 3 distinct samples. The images (~5x5 mm²) show the visual color change of the hydrogels as a function of increasing extent of phosphorylation.

Raw peak diffraction spectra as a function of PKA concentration in phosphorylation reaction

Figure S3. Red shift in peak optical reflectance of hydrogel-encapsulated CCAs with increasing PKA concentration. The PKA treatment was performed at concentrations ranging from 0 (black curve) to 25 U/µL (brown curve) for 2 h at 30 °C. The raw diffraction spectra shown here correspond to the dose-dependent measurements presented in Fig. 3b.
Detector response in the presence of mobile charged small molecules

A potential concern for using the photonic crystal biosensor for screening kinase inhibitors and activators is that charged, small molecules present during the phosphorylation reaction may affect the optical response of the sensor. To address this concern, the response of the sensor to phosphorylation in the presence and absence of charged small molecules was investigated. In this case, we utilized biologically relevant amino acids (lysine for the positively-charged molecule and glutamic acid for the negatively-charged molecule) at 10 mM concentrations that are well above relevant concentrations for compounds for inhibiting and activating kinases. The results, reported in Fig. S4, indicate that the charged molecules have no effect on the final optical response of the sensor. Exogenous charged and uncharged species are removed upon extensive rinsing in ultrapure water, as is always done prior to optical characterization of the biosensor, thereby preventing interference from these compounds.

![Figure S4](image)

**Figure S4.** Optical response of the photonic crystal biosensors to kinase activity in the presence of positively- and negatively-charged small molecules. The hydrogel-encapsulated CCAs were kinase treated alone, with 10 nM positively-charged lysine, or with 10 nM negatively-charged glutamic acid. All samples were incubated with 16 U/µL PKA for 2 h at 30 °C in 50 mM tris-HCl buffer, at pH 7.5, with 10 mM MgCl₂ and 1 mM ATP. Error bars represent ±1σ from the mean over 3 samples. The similarity in red shift in peak diffraction between samples indicated that exogenous charged molecules in the kinase reaction step may readily be washed from the hydrogel samples and, as such, do not alter the extent of hydrogel swelling.
**Extent of phosphorylation as a function of time and enzyme concentration as determined by theoretical fitting**

The time course and dose response data in Fig. 3 can be fit with the theoretical model of swelling in ionic polymer networks, using a single fitting parameter, as indicated with the solid curves. The model assumes that the change in charge concentration varies linearly with both time and enzyme activity, which is consistent with Michaelis Menten kinetics at substrate saturating and initial rate conditions. Assuming substrate saturation ([S] \(\gg\) \(K_m\)), plots of product ([P]) as a function of time (t) and [P] as a function of enzyme concentration ([E]) are shown in Fig. S5.6 The slope of [P] versus t in Fig. S5a is the activity (d[P]/dt) and, at this condition, is equivalent to \(V_{max}\). In Fig. S5b, the slope of [P] versus [E] is equivalent to \(k_{cat} \times t\), from which \(k_{cat}\) was determined to be 2.0 s\(^{-1}\). The same value of \(k_{cat}\) could also be calculated independently from the plot of [P] versus t using the known value of [E] (16 U/µL).

**Figure S5.** Extent of phosphorylation as a function of a) time upon treatment with 16 U/µL of PKA and b) PKA concentration for 2 h treatments. Experimental measurements are shown as blue points, the solid black curves represent model predictions over the range fitted, and the dashed curves are extrapolations from the model.
Application of the photonic crystal biosensor to the detection of phosphatase activity

We have also demonstrated that the photonic crystal biosensor can be used to detect the reverse (i.e., dephosphorylation) reaction, involving the removal of immobilized negative charges, by phosphatases. Figure S6 shows the spectral data for a CCA biosensor functionalized with 45 mol% phosphorylated peptide and 55 mol% non-phosphorylated peptide before (black curve) and after (blue curve) treatment with Antarctic phosphatase (0.1 U/µL at 37 °C for 16 h). After incubation of the sample with the phosphatase, an ~30 nm blueshift in the wavelength of peak diffraction was observed. In addition, the recyclability of the biosensor was demonstrated by subsequent phosphorylation upon incubation with PKA (16 U/µL at 30 °C for 3 h), leading to a red shift in the wavelength of peak diffraction similar to that quantified in Fig. 3a (an ~60 nm red shift).

Figure S6. Optical detection of phosphatase activity using the photonic crystal biosensor. The hydrogels were functionalized with a 100 mM peptide solution that contained 45 mol% phosphorylated and 55 mol% non-phosphorylated peptide. The black curve is the spectral data of the sample post-peptide functionalization and the blue curve is the same sample after incubation with 0.1 U/µL Antarctic phosphatase at 37 °C for 16 h. Control samples showed no shift in peak diffraction upon phosphatase treatment.

IV. References

(1) Reese, C. E.; Guerrero, C. D.; Weissman, J. M.; Lee, K.; Asher, S. A., J Colloid Interface Sci 2000, 232 (1), 76-80.
(2) Hermanson, G. T., Bioconjugate techniques. 2nd edition. ed.; p.219-223.
(3) Nakajima, N.; Ikada, Y., Bioconjugate Chem 1995, 6 (1), 123-130.
(4) Uchida, E.; Uyama, Y.; Ikada, Y., Langmuir 1993, 9 (4), 1121-1124.
(5) Flory, P. J., Principles of polymer chemistry. Cornell University Press: Ithaca., 1953; p 584-589.
(6) Hemmer, W.; McGlone, M.; Tsigeln, I.; Taylor, S. S., J Biol Chem 1997, 272 (27), 16946-16954.