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Detection of growth factor binding to gelatin and heparin using a photonic crystal optical biosensor

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ABSTRACT

Drug–carrier interactions are important to protein controlled release systems to protect the protein from denaturation and ensure properly timed release. A novel photonic crystal biosensor was used to investigate a gelatin–protein controlled release system to determine the amount of protein bound to the carrier at physiological conditions. The Biomolecular Interaction Detection (BIND) system reflects a narrow band of wavelengths when white light is shone incident to the grating. As mass is deposited onto the surface, the peak wavelength value is shifted due to changes in the optical density of the biosensor. The BIND system was used to detect the binding of growth factors onto acidic gelatin, basic gelatin, and heparin on the sensor surface. Through a series of experiments, including functionalizing the sensor, adjusting the ionic strength of the solution, adjusting the substrate concentration, and minimizing non-specific signal, the adsorption of the gelatins and heparin on the sensor was enhanced. The binding interaction of recombinant human transforming growth factor (rhTGF)-β1 and bone morphogenetic protein (rhBMP)-2 with the two types of gelatin and heparin were investigated. The strength of the interaction between rhTGF-β1 and the substrates is in the following order: heparin>acidic gelatin>basic gelatin. RhBMP-2 bound to the substrates but with less intensity than TGF-β1: heparin>basic gelatin>acidic gelatin. This work provides support for the controlled release mechanism through degradation of the gelatin carrier.

1. Introduction

Gelatin is a commonly used polymer in pharmaceutical and food industries [1]. Two forms of gelatin, acidic (isoelectric point (pI) = 5.0) and basic (pI = 9.0), have been used successfully as delivery vehicles for proteins involved in tissue regeneration [2–7]. Both types have been shown to interact with specific growth factors through ionic complexation (acidic gelatin with transforming growth factor (TGF)-β1 [8,9]) or hydrophobic interactions (basic gelatin with bone morphogenetic protein (BMP)-2 [7,9]). Protein–gelatin interactions encourage protein stability and improve controlled release as large molecules are not effectively released by diffusion alone [10]. Once complexed, the proteins are released through enzymatic degradation of the gelatin rather than through simple diffusion, typically a more rapid process. Therefore, controlled release can be achieved by adjusting the gelatin cross-linking density to tailor the gelatin degradation rate.

Acidic gelatin hydrogel sheets have been used to deliver TGF-β1 to a variety of bony defects [5,11,12]. Basic gelatin microspheres and sheets have been used to deliver BMP-2 [6,13]. Measurements of ^{125}I-TGF-β1 and ^{125}I-BMP-2 absorptions into gelatin hydrogels have been investigated [6,7,12]. The ^{125}I-TGF-β1 (pI = 10.3) readily interacts with acidic gelatin while ^{125}I-BMP-2 (pI = 8.5) shows only a slightly higher affinity for basic gelatin versus the acidic type [7]. *In vivo* release profiles of the labeled proteins reveal no therapeutic activity, suggesting that the label inactivates the proteins [5–7,12].

Solution techniques work well for measuring drug–vehicle affinities in drug delivery systems where the drug is incorporated during the vehicle fabrication process. However, when the drug is added after the vehicle fabrication a technique capable of detecting surface interactions is needed. Those who use such surface techniques have noted that the loss of rotational and diffusional freedom can affect the binding kinetics and reaction thermodynamics [14–16]. For these reasons, surface type sensors may provide a more accurate measurement of the interactions leading to the controlled release of the proteins than solution systems. Within the gelatin drug delivery system presented here, the protein is added after the gelatin is cross-linked not while the gelatin is in solution.

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Label-free photonic crystal biosensors have recently been shown to be a highly sensitive method for performing a wide variety of biochemical assays [17,18]. An advantage of using label-free biosensors is that a label-free detection method removes experimental uncertainties associated with the effects of labels on molecular conformation, blocking of active binding epitopes, steric hindrance, and the inability to find available labels for specific molecules [18–20]. By removing labels, the experiment is simplified and the material cost is reduced.

The Biomolecular Interaction Detection (BIND) system (SRU Biosystems, Woburn, MA) utilizes a sub-wavelength grating structure that when illuminated with a broadband light source, reflects only a narrow resonant wavelength band [20]. The resonant peak wavelength value (PWV) is shifted positively as molecules attach to the sensor surface. The surface optical density of the biosensor changes with the addition of molecules on the surface, enabling the measurement of bound biomolecules without the use of any labels. The grating is attached to the bottom of a 96-well microtiter plate that allows for high-thorough put testing of the protein–gelatin interactions. The BIND system has demonstrated the capability of resolving surface molecular density changes to 0.1 pg/mm^2 [19].

In the present work, the BIND system was used to detect the binding of growth factors onto acidic gelatin, basic gelatin, and heparin on the sensor surface. The molecule immobilized on the sensor will be referred to as the "substrate" and the molecule in solution will be referred to as the "ligand". Through a series of experiments, including functionalizing the sensor, adjusting the ionic strength of the solution, adjusting the substrate concentration, and minimizing non-specific signal, the adsorption of the gelatins and heparin on the sensor was enhanced. Once substrate adsorption was maximized, the binding interaction of rhTGF-β1 and rhBMP-2 with two types of gelatin and heparin were investigated.

2. Materials and methods

Pharmaceutical grade acidic (pI = 5.0) and basic (pI = 9.0) gelatins were kindly provided by Nitta Gelatin Corp. (Osaka, Japan). Carrier-free rhBMP-2 and rhTGF-β1 were purchased from R&D Systems (Minneapolis, MN). Heparin, phosphate buffered saline (PBS, pH = 7.4), and all other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. TiO_2 microtiter plates and glass slides with and without aldehyde functionality were kind gifts from SRU Biosystems, Inc (Woburn, MA).

2.1. Optimization of BIND for use with gelatin and heparin

The BIND system had not previously been tested with gelatin; therefore several experiments were conducted to optimize the system for investigating protein binding to gelatin. In general for each test, the microtiter 96-well plate was first washed with deionized (DI) water. Then fresh DI water was added to each well and the baseline PWV was recorded until the signal was stable. The water was removed and the substrates were added to the wells. The immobilization of the substrate was measured by the shift in PWV for at least 30 min after addition and the plate incubated at 4 °C overnight. The plates were warmed at room temperature and a final measurement of the stable substrate layer was collected the next morning. The wells were washed with DI to remove any unbound substrate and the PWV shift recorded until equilibrium was established.

Acidic gelatin, basic gelatin, and heparin were added to a plain TiO_2 plate and to a TiO_2-aldehyde plate in the manner described to determine which plate provided sufficient immobilization for use in the study. Once the plate was chosen, the pH of the gelatin solutions was adjusted from 3 to 11 to achieve optimal binding conditions. The concentration of each substrate was adjusted from 0.01 to 0.5 mg/mL to determine the optimal concentration to create a stable signal. From these trials, the optimal condition for each substrate was identified for the subsequent protein–gelatin binding tests.

2.2. Thickness of the gelatin layer

Atomic force microscopy (Asylum Research MFP-3D) was used to measure the thickness of the gelatin layer on the TiO_2. Aldehyde functionalized sensors were mounted on glass slides and a thin layer of gelatin was created on the surface by immersing the slide in a 0.05 mg/mL acidic gelatin solution at 4 °C overnight. The slide was rinsed with deionized water and allowed to soak in fresh DI water until scanned. Contact mode with a Budget Sensors tip (Innovative Solutions Bulgaria Ltd.) was used to create a scratch on a flat portion of the sensor through the gelatin layer to the TiO_2. The scratch was then scanned under tapping mode to measure the thickness of the gelatin layer.

2.3. Binding of growth factors

Once the substrate was properly bound, a blocking agent (Sea Block™) was added to prevent non-specific binding to the sensor surface. A separate set of experiments was used to determine the optimal blocking agent and protein concentration using bovine serum albumin as a model protein [21]. Carrier-free rhBMP-2 and rhTGF-β1 were reconstituted in phosphate buffered saline (PBS, pH = 7.4) to a final concentration of 0.005 mg/mL. PBS was used to wash the blocking agent prior to the addition of each protein. Five wells were used for each protein. The amount of protein bound on the surface was normalized to the amount of substrate present in the same well and reported as the endpoint. A conversion factor of 111 ng/1 nm was used to approximate the mass of protein bound to the surface [22].

2.4. Statistical analysis

One-way analysis of variance (ANOVA) was performed using a statistical software (Minitab™). Fisher's pairwise comparison was used to determine the significance between groups. A p value of 0.05 or less was considered significant. Results are reported as the mean ± standard error.

3. Results

3.1. Optimization of the BIND system

Both gelatins bound strongly to the original TiO_2 surface as indicated by the large shift in the PWV (Fig. 1), with more basic gelatin binding than the acidic gelatin. However, the heparin did not bind to the plain sensor but instead bound readily to the aldehyde treated plate. Both gelatins also bound to the TiO_2-aldehyde plate, with the acidic gelatin binding more strongly than the basic gelatin. Thus, the TiO_2-aldehyde plate was chosen for all future experiments.

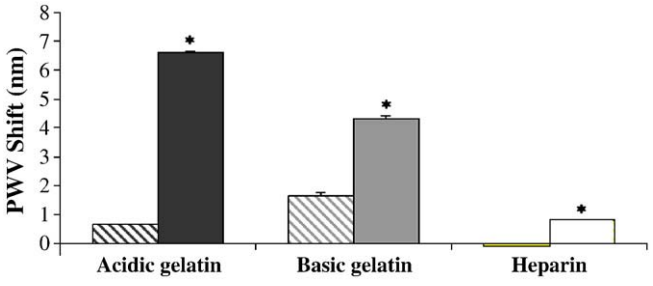

Fig. 1. Binding of acidic (■) and basic (▩) gelatin and heparin (□) to the BIND 96-well plate with (solid bars) or without (striped bars) aldehyde functional groups. Each substrate concentration was 0.5 mg/mL. The functionalized plate was used for all future studies (*).

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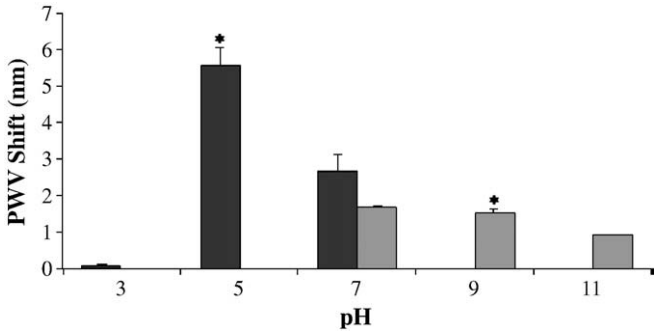

Fig. 2. Acidic (■) and basic (▩) gelatin binding (0.5 mg/mL) at various pH conditions on aldehyde functionalized plates. Conditions chosen for future studies are denoted with asterisks (*).

The pH of each gelatin solution was adjusted to improve binding. The largest PWV shift was observed when acidic gelatin was at its pI = 5 (Fig. 2). Basic gelatin's binding increased slightly as the pH was decreased. With very little significant binding difference between pH 7.0 and 9.5, the pI of the basic gelatin was chosen for all future experiments.

Concentration trials revealed increasing binding with increasing concentration for each substrate (Fig. 3). The optimal concentration was 0.05 mg/mL for acidic gelatin and 0.5 mg/mL for basic gelatin. The amount of bound heparin increased only slightly as the concentration increased. Therefore, for the best comparison, two heparin concentrations were chosen: 0.05 and 0.5 mg/mL.

3.2. Thickness of the gelatin layer

The gelatin created a smooth layer over the TiO_2 decreasing the surface roughness from 962 pm to 618 pm root mean squared roughness (Fig. 4). The layer conformed to the optical grating and increased the width of the grating struts. Several 100 nm scratches were created in the space between sensor areas to measure the gelatin layer thickness (Fig. 5). From line scans across the scratches, ignoring the debris, the gelatin layer was determined to be approximately 40 nm.

3.3. Binding of growth factors

A concentration test with rhTGF-β1, confirmed that 0.005 mg/mL was the lowest concentration that still provided a detectable signal (data not shown). Both rhTGF-β1 and rhBMP-2 bound to heparin (Figs. 6 and 7), with little difference in the amount bound to the two

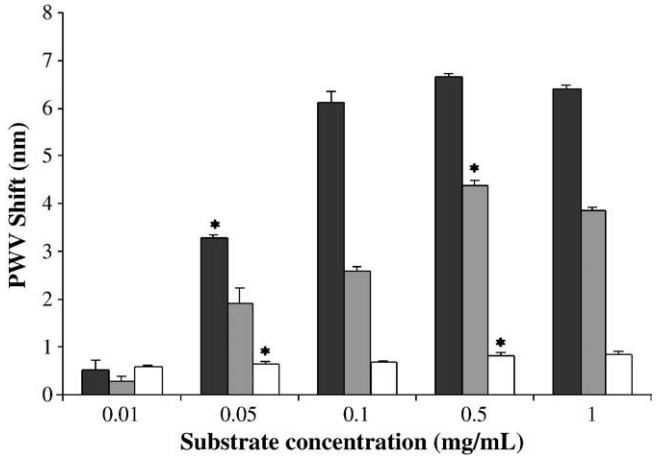

Fig. 3. Binding of acidic (■) and basic (▩) gelatins and heparin (□) to the aldehyde functionalized sensor. Asterisks (*) denote final concentration used for each substrate.

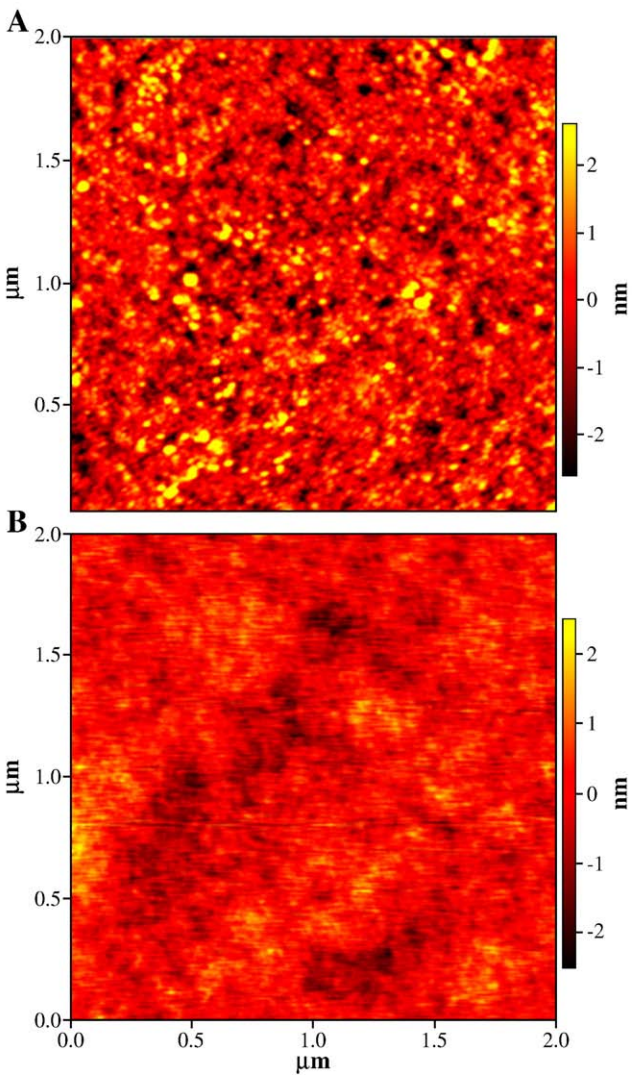

Fig. 4. Representative atomic force microscopy images of the TiO_2-aldehyde sensor surface before (A) and after (B) the addition of 0.05 mg/mL acidic gelatin. The granular structure of the sputter coated TiO_2 (A) with a roughness of 962 pm was smoothed to 618 pm by the addition of gelatin (B).

concentrations. For rhTGF-β1, the order of binding interaction was: heparin > acidic gelatin > basic gelatin. The same mass of growth factor was deposited on the surface of acidic gelatin and heparin (Table 1); however, when normalized with the mass of substrate per well, the interaction between rhTGF-β1 and heparin was greater than that with acidic gelatin (Fig. 6). More rhTGF-β1 was deposited on the acidic gelatin (54.4 ± 2.1 ng) than the basic gelatin (27.8 ± 3.3 ng).

RhBMP-2 displayed a lower affinity for all substrates used than the rhTGF-β1. The binding order for rhBMP-2 was heparin > basic gelatin > acidic gelatin. A greater mass of protein was deposited on the basic gelatin (21.1 ± 1.8 ng) than the heparin (15.5 ± 1.2 ng) and the acidic gelatin (8.8 ± 1.5 ng). Again, with normalization, it was apparent that the rhBMP-2 interacts more strongly with heparin than with basic or acidic gelatins (Fig. 7).

4. Discussion

The BIND system, once optimized, reproducibly measured protein-binding phenomenon on a gelatin-coated surface. This platform was chosen because the protein is added after the hydrogel has been formed not while the gelatin is in solution. Solution measurements would provide the maximum interaction between free gelatin chains

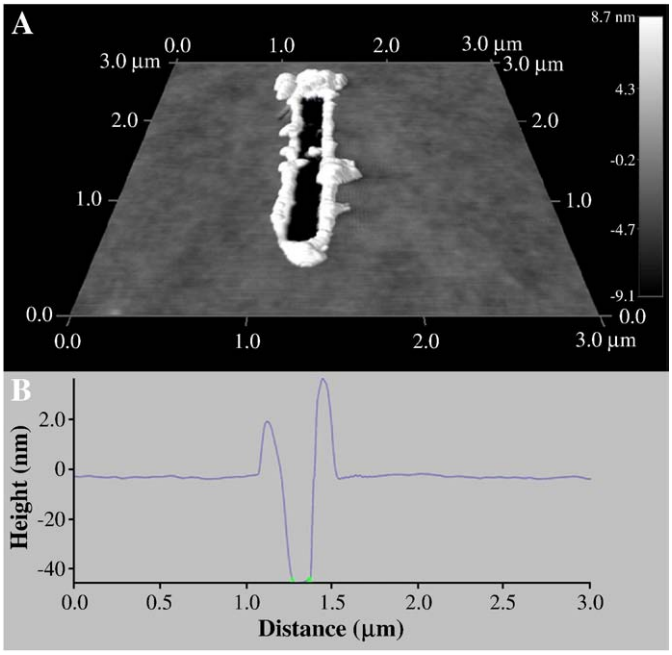

Fig. 5. Atomic force micrograph of a representative scratch (A) created in the gelatin layer to measure its thickness. Line profiles (B) across the scratch reveal a flat bottom, which indicates the scratch was deep enough to reveal the TiO_2 layer below. The gelatin layer was approximately 40 nm thick.

and free growth factor. Yet, in a hydrogel, the gelatin chains would be limited in movement due to cross-linking, effectively decreasing the binding sites available to the proteins. Thus, the gelatin–protein interaction will be reduced. In addition, the BIND system does not require the protein to be labeled that could possibly interfere with the protein–gelatin interactions.

To optimize the BIND system for gelatin–protein interaction measurements, several choices were made. First the TiO_2-aldehyde plate was chosen for optimal heparin and gelatin binding. The smaller heparin molecule did not bind to the plain TiO_2 plate but bound well to the functionalized surface. As the gelatin hydrogel is cross-linked with glutaraldehyde, using the TiO_2-aldehyde plate recreates this condition and further constrains the gelatin on the surface. The pH and concentration of each substrate solution was adjusted for further optimization. The final pH chosen for each gelatin was equal to each pI. When the pI is equal to the pH, a maximum protein adsorption on a surface should be observed [23]. In the acidic gelatin, a clear increase in binding is observed at its pI while the basic gelatin appears to plateau as the pH is decreased. The plateau could be affected if high concentration of basic gelatin was used and the pH did not exactly

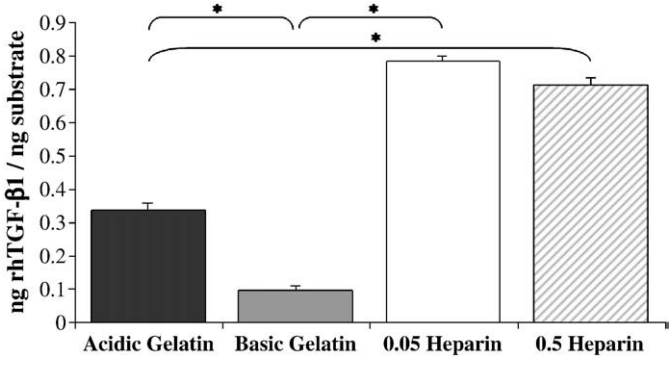

Fig. 6. RhTGF-β1 binding to various substrates normalized to the amount of substrate. Asterisks (*) denote statistical significance between groups ($p<0.05$).

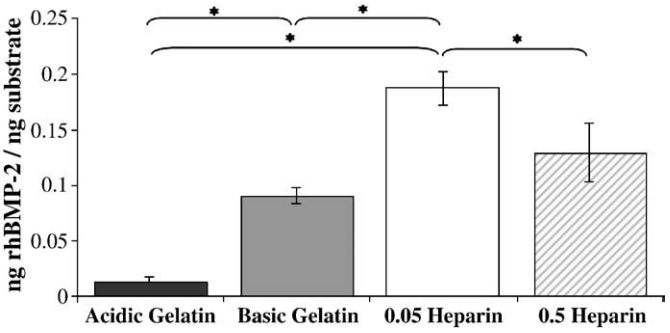

Fig. 7. RhBMP-2 binding normalized to various substrates. Asterisks (*) denote statistical significance ($p<0.05$) among the substrates.

equal the pI, as this could decrease the binding to the surface. A lower concentration of acidic gelatin (0.05 mg/mL) was used to achieve a comparable PWV shift signal to the basic gelatin (0.5 mg/mL). The heparin concentration was equal to each substrate for ease of protein-binding comparison.

Due to the inherent difficulties of interpreting kinetic data when multiple binding sites are available [16], only endpoint analysis was performed. For the growth factors of interest, a percentage of each protein was irreversibly bound to the gelatin at physiological conditions. This finding supports the *in vivo* reports of the protein release rate being driven by the gelatin degradation rate as an irreversibly bound drug would exhibit this type of behavior. A burst release is typically seen *in vivo* with microsphere delivery systems. This burst can be attributed to unbound growth factor as a similar amount of protein was removed by washing in the BIND system [7,8]. The burst effect could be reduced or eliminated by washing the gelatin hydrogel after growth factor binding has occurred.

The BIND system further confirms the use of acidic gelatin as the appropriate carrier for rhTBF-β1 and basic gelatin for rhBMP-2. The results are comparable to values found in the literature with approximately 40% rhTGF-β1 binding to acidic gelatin and 20% rhBMP-2 binding to basic gelatin [7]. The slightly basic pI of rhBMP-2 would suggest that the rhBMP-2 would favor acidic gelatin over basic gelatin. But this is not seen experimentally. It has been suggested that the interaction between BMP-2 and gelatin are more physicochemical, intermolecular forces such as London Dispersion forces, dipole–dipole interactions, and hydrogen bonding, than electrostatic interactions [7,24,25]. Various growth factors are known to interact with physicochemical or intermolecular forces in order to maintain biological functions in the body [7,26]. In this work, due to the fact that no changes in BMP-2–gelatin interactions have been observed during the increase of ionic strength, electrostatic interactions is ruled out [7]. The weakness of the interaction also indicates physicochemical interaction, whereas ionic or covalent binding would yield higher binding signals [27,28]. RhBMP-2 is a glycosylated protein. It has been

Table 1
Mass of growth factor bound to each substrate after the final wash. Results are presented as mean ± standard error.

	rhTGF-β1			rhBMP-2		
	Amount (ng)	% Bound	ng Protein per ng substrate	Amount (ng)	% Bound	ng Protein per ng substrate
Acidic gelatin	54.4 ± 2.1	36.3 ± 1.4	0.34 ± 0.02	8.8 ± 1.5	5.8 ± 1.0	0.01 ± 0.01
Basic gelatin	27.8 ± 3.3	18.5 ± 2.2	0.1 ± 0.01	21.1 ± 1.8	14.1 ± 1.2	0.09 ± 0.01
0.05 Heparin	45.5 ± 0.5	30.3 ± 0.4	0.79 ± 0.01	15.5 ± 1.2	10.4 ± 0.8	0.19 ± 0.01
0.5 Heparin	47.7 ± 1.0	31.8 ± 0.7	0.71 ± 0.02	8.7 ± 1.6	5.7 ± 1.1	0.13 ± 0.03

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reported that these sugar residues interfere with the protein binding effectively to gelatin [7]. The BIND system provides further evidence of rhBMP-2's higher affinity for basic gelatin than acidic gelatin, yet still a weaker interaction than those seen with other proteins.

Higher binding signals of both growth factors to heparin compared to the gelatins were expected. Numerous reports have described high binding affinity of heparin to both (rhTGF)-β1 [29–31] and (rhBMP)-2 previously [29,32–34], thus results of the experiments further confirm the interactions between heparin to the growth factors, and validated the photonic crystal label-free method.

5. Conclusions

The use of a photonic crystal optical biosensor successfully measured surface binding phenomenon of several proteins on gelatin and heparin. The proteins were prohibitively expensive in the quantities required for solution interactions (rhTGF-β1 $81/μg and rhBMP-2 $45/μg). The BIND system utilized smaller volumes with the ability to measure several interactions under the same conditions simultaneously. This system was optimized for substrate binding on a TiO_2-aldehyde treated sensor while using a blocking agent, Sea Block™, to eliminate non-specific binding. In addition, photonic crystal label-free detection method was validated through the detection of high binding signal of both growth factors binding to heparin.

Binding between rhTGF-β1 and acidic gelatin was significantly higher than on basic gelatin. RhBMP-2 exhibited higher binding to basic gelatin than acidic but bound amounts were lower than rhTGF-β1 on the two gelatins. Both proteins bound irreversibly to the gelatins and the amount of unbound protein corresponds to the burst release observed *in vivo*. Growth factor concentration tests could identify the saturation point and be used to optimize growth factor incorporation into gelatin delivery systems. Currently, it appears necessary to wash the unbound protein from the gelatin delivery system to eliminate this burst release in the future. This work provides significant support for the hypothesis that the gelatin degradation rate controls the release rate of the bound protein.

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