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# Photoimmobilization of biomolecules within a 3-dimensional hydrogel matrix

X. CAO [1,3,*] and M. S. SHOICHET [1,2,3,†]

[1] *Department of Chemical Engineering and Applied Chemistry, University of Toronto, 4 Taddle Creek Road, Toronto, Ontario, M5S 3G9, Canada*
[2] *Department of Chemistry, University of Toronto, 4 Taddle Creek Road, Toronto, Ontario, M5S 3G9, Canada*
[3] *Institute of Biomaterials and Biomedical Engineering, University of Toronto, 4 Taddle Creek Road, Toronto, Ontario, M5S 3G9, Canada*

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**Abstract**—It has been recognized that a three-dimensional cell invasive scaffold that provides both topographical and chemical cues is desirable in regenerative tissue engineering to encourage cell attachment, migration, regrowth and ultimately tissue repair. Carbohydrate hydrogels are attractive for such applications because they are generally biocompatible and able to match the mechanical properties of most soft tissues. Although carbohydrate hydrogels have been previously modified with cell adhesive peptides and proteins, complicated hydrogel matrix activation was required prior to biomolecule coupling and, perhaps more importantly, the overall immobilization yield was low at ~1%. In this study, we report the photo-immobilization of a model biomolecule, ovalbumin (OVA), to agarose gel. We describe two methods of modification where the photoactive moiety is coupled to either the protein (i.e. OVA) or the matrix (i.e. agarose) prior to immobilization. We found that the photo-immobilization yield depends on the location of the photoactive moiety. Using photoactive OVA, 1.8% of the OVA initially incorporated into the agarose gel is immobilized; using photoactive agarose, 9.3% of the OVA initially mixed with the agarose is immobilized. The latter is a significant improvement over previous yields and may be useful in attaining our goal of immobilizing a biomolecule gradient for guided tissue regeneration.

*Key words*: Hydrogel; photochemistry; benzophenone; immobilization; nerve regeneration; tissue engineering; 3D scaffold.

*Current address: Center for Engineering in Medicine, Harvard Medical School, Massachusetts General Hospital, Shriners Burns Hospital, 51 Blossom Street, Boston, MA 02114, USA.

†To whom correspondence should be addressed. Phone: 416-978-1460. Fax: 416-978-4317. E-mail: molly@ecf.utoronto.ca

## INTRODUCTION

Cells respond to their local environment, including both topographical features, such as three-dimensional (3D) organization of neighboring matrix textures [1], and chemical features, such as cues from fibrillar protein networks known as extracellular matrix (ECM) [2]. For regenerative tissue engineering, a three-dimensional cell invasive biomaterial scaffold that provides both chemical and topographical cues is critical for cell migration, regeneration and tissue organization. For example, we showed that a 3D biodegradable scaffold enhanced bone regeneration *in vitro* [3] and others have shown that a cell permissive hydrogel matrix [4], within a hollow fiber membrane, promoted nerve regeneration between two severed nerve ends.

Hydrogels, such as agarose, are particularly attractive for use in soft tissue engineering applications because they are predominantly biocompatible [4]. Being inert to protein adsorption and physically stronger than most of the non-chemically-crosslinked hydrogels at the same concentration, agarose has been widely used as a packing material in chromatography (e.g. Sepharose) [5–7] and as a model hydrogel matrix to study cell permissive scaffolds for potential applications in regenerative tissue engineering [4]. Furthermore, because agarose is thermally reversible, it can be molded into different geometries for specific tissue engineering applications.

Several strategies have been pursued to modify agarose via the hydroxyl groups. For example, biomolecules were covalently bound to cyanogen bromide-activated hydroxyl groups [8–10]; however, because cyanogen bromide is highly toxic, 1,1′-carbonyldiimidazole (CDI) activation is now more common. Although CDI activation resulted in a high degree of both activation and biomolecule immobilization yield [6, 11], it also resulted in crosslinking, which affected the thermal reversibility of agarose [12]. This has limited the utility of CDI activation for tissue engineering applications. Alternatively, UV irradiation has been applied to photoimmobilize cell adhesive peptides to agarose matrices. For example, Borkenhagen *et al.* [13] derivatized CDPGYIGSR with the photoactive benzophenone (BP) and immobilized the photoactive polypeptide conjugate to agarose matrices by UV irradiation in situ. This approach took advantage of relatively simple photochemistry to immobilize biomolecules to a 3D agarose gel, thereby providing an alternative way to derivatize hydrogel matrices for applications in tissue engineering. Unfortunately, this approach resulted in a low immobilization yield of ~1% [12].

We recently reported the preparation of a well-defined diffusible neurotrophic factor concentration gradient in a three-dimensional agarose gel for guided neurite outgrowth [14]. We believe that an immobilized neurotrophic factor concentration gradient will also direct neurite outgrowth in a manner similar to the diffusible NGF concentration gradient, based on recent published data [15, 16]. While others have immobilized biomolecule concentration gradients on two-dimensional surfaces [17, 18], no one has immobilized a three-dimensional gradient, to the best of our knowledge. Creation of a 3D gradient is one of our ultimate goals, but before we could attempt this, we had to first determine a method to increase the

immobilization yield beyond the previously reported 1% [12]. We chose to use ovalbumin (OVA), as our model biomolecule, to study immobilization.

Specifically, we compared the efficiency of two UV irradiation approaches to immobilize OVA within a 3D agarose gel matrix. By modifying OVA with the photoactive BP prior to immobilization in agarose, we were able to achieve an immobilization yield of 1.8%. By modifying agarose with BP prior to immobilizing OVA, we were able to increase the immobilization yield to 9.3%. The amount of protein immobilized to a substrate, due to BP photolysis, is almost directly proportional to that initially incorporated [19]. We have chosen to report the percentage of molecules immobilized as the measure of photoimmobilization yield because our ultimate objective is to immobilize a concentration gradient of neurotrophic factors in a three-dimensional hydrogel matrix to promote and guide nerve regeneration.

## EXPERIMENTAL

### *General considerations*

Two different approaches were taken to photoimmobilize OVA to a 3D agarose matrix: (1) photoactive OVA and (2) photoactive agarose, as shown is Fig. 1.

### *Materials and methods*

All chemicals were purchased from Sigma (St. Louis, MO) and used as received, unless otherwise indicated. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC) was purchased from Pierce (Rockford, IL) and used as received. Deionized distilled water was obtained from a Millipore Milli-RO 10 Plus and Milli-Q UF Plus (Bedford, MA) and used at 18 MΩ resistance.

### *Photoactive OVA approach*

In this approach, OVA was first modified with the photoactive BP, then OVA-BP was dispersed into an agarose hydrogel and photoimmobilized in situ by UV-irradiation.

### *Preparation of photoactive OVA*

OVA was modified with benzophenone (BP), by reacting the primary amine groups of OVA with 4-benzoylbenzoic acid succinimide ester (NHS-BP). In a typical reaction, a predetermined amount of NHS-BP was dissolved into 2 ml of *N*,*N*-dimethylformamide (DMF, anhydrous), to which 10 ml of 2.15 mg/ml (w/v) OVA in phosphate-buffered saline (PBS, pH 7.4) solution was added dropwise with stirring. The reaction was carried out at 4°C under continuous agitation for a predetermined duration and was quenched by adding 50 $\mu$l ethanolamine. The resulting product (i.e. photoactive OVA-BP) was purified using 3000 molecular weight cut-off dialysis tubing (Spectrum, CA) against PBS. The bath was periodically changed

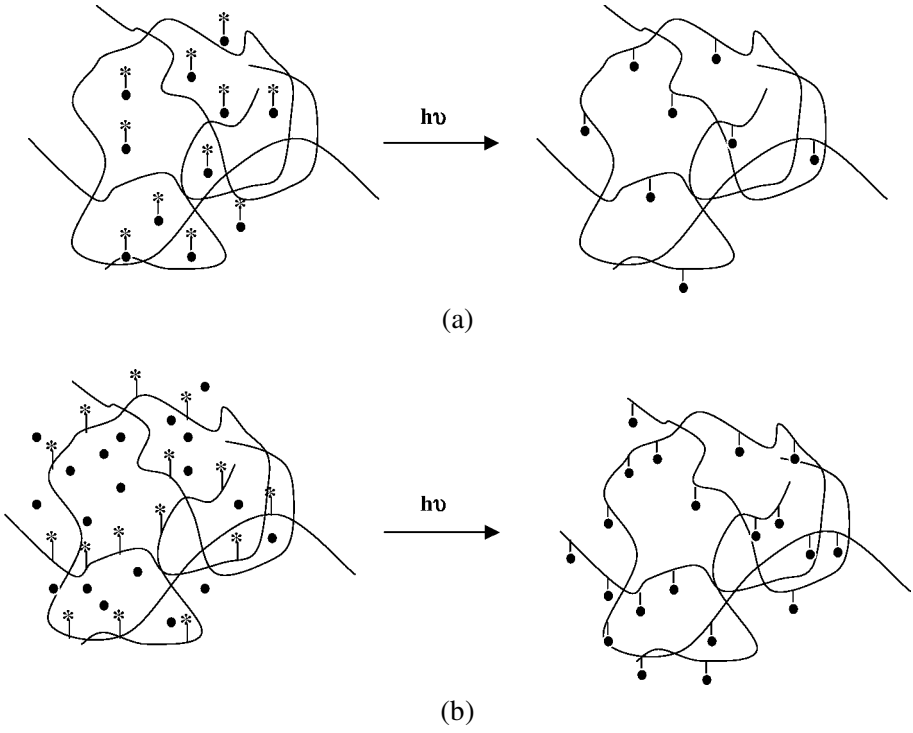



**Figure 1.** Two approaches were used to photoimmobilize biomolecules to agarose: (a) in the photoactive biomolecule (OVA) approach, the photoactive moiety (*) is conjugated with the biomolecule (●) prior to immobilization; and (b) in the photoactive agarose approach, the photoactive moiety (*) is covalently bound within the agarose gel prior to biomolecule (●) photo-immobilization.

and monitored by UV-vis spectrophotometry until there was no (appreciable) absorbance at 260 nm associated with unreacted NHS-BP. All procedures were performed under subdued lights to avoid any unintended photoreaction. Two parameters, the molar ratio of NHS-BP to OVA and the reaction time, were varied to control the degree of modification of OVA-BP. Reactions were conducted at NHS-BP to OVA molar ratios of 1 : 1, 2 : 1, 10 : 1, 20 : 1, 40 : 1, and 60 : 1 for 12 h. In a separate set of experiments, reactions were performed for 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, and 36 h for a NHS-BP to OVA molar ratio of 20 : 1.

*Estimation of the degree of substitution*

The degree of substitution of OVA by NHS-BP was estimated according to Stocks *et al.* [20], by determining the unreacted primary amine groups of OVA using a fluorimetric assay. Briefly, photoactive OVA-BP was dissolved in PBS solution and was serially diluted to 0.3 mg/ml, 0.15 mg/ml, 0.075 mg/ml and 0.0375 mg/ml. One half ml of fluorescamine in acetone (0.3 mg/ml, w/v) was added to 1.5 ml of OVA-BP solution at each concentration. The mixture was then mixed by vortex. After 8 min, the fluorescence in the mixture was measured on a fluorimeter (Turner,

Model 450) with an excitation wavelength of 360 nm and emission at 420 nm. Identical procedures were repeated for unmodified OVA to determine the total titratable amine content.

*Radioactive labeling of photoactive OVA*

Tyrosine residues of photoactive OVA (OVA-BP) were labeled as previously described with radioactive iodine ($^{125}$I) [21]. Briefly, 6 ml of OVA-BP in PBS solution (2 mg/ml) was reacted with 1 mCi of carrier-free Na $^{125}$I (Amershan Pharmacia Biotech, QC, Canada) in the presence of Iodobeads (Pierece, Rockford, IL) for 15 min. Free iodide was removed by successive passes through columns packed with anion-exchange resin (Dowex 1-X8, Aldrich). The radiolabeled OVA-BP was used to determine the photo-immobilization yield of OVA-BP in the agarose matrix.

*Determination of the duration of the photo-irradiation*

In order to determine the appropriate duration of photo-irradiation, photoactive OVA-BP was dissolved in PBS and was exposed to UV irradiation at 355 nm at different exposure times. The UV-vis spectra of the irradiated OVA-BP were monitored, and the gradually reduced absorbance at 260 nm associated with BP photolysis upon UV irradiation was used to determine the appropriate UV-irradiation time.

*Determination of photoimmobilization yield of OVA-BP*

One ml of 2% agarose solution was mixed with 1 ml of I-125 radiolabeled OVA-BP in PBS solution (2 mg/ml) to form 2 ml of a 1% agarose solution. The resulting solution was cast into a 35 mm Petri dish and allowed to set at room temperature overnight. This procedure formed a 2 mm thick gel disk (hydrated state) that was then cut into pieces and separately weighed. The agarose/benzophenone-modified OVA disks (OVA-BP-1) were irradiated at 355 nm for 45 min using a UV reactor equipped with 8 mercury lamps (Rayonet, Branford, CT). These samples were washed with washing buffer (0.1 M sodium bicarbonate and 0.1 M sodium iodide) until the radioactivity of the buffer was less than twice that of the background noise. Radioactivity was measured by a LKB 1282 Compugamma universal gamma counter (Fisher Scientific). Two additional agarose/OVA-benzophenone disks served as controls: (1) where an identical disk was not irradiated but was washed, as described above (OVA-BP-2) and (2) where an identical disk was neither irradiated nor washed (OVA-BP-3).

The radioactivities in all three agarose/OVA-benzophenone disks (i.e. OVA-BP-1, OVA-BP-2 and OVA-BP-3) were then measured. Comparisons between the weight normalized radioactivities among the three disks were used to determine both the photoimmobilization yield (i.e. OVA-BP-1 vs. OVA-BP-3) and any nonspecific binding (i.e. OVA-BP-2 vs. OVA-BP-3).

*Photoactive agarose approach*

In this approach, the photoactive moiety (i.e. BP) was immobilized to the agarose matrix first, and then native (or unmodified) OVA was photoimmobilized to the matrix.

*Preparation of photoactive poly(allylamine) [PA-BP]*

A 0.75 ml solution of 0.035 M NHS-BP in *N*,*N*-dimethylformamide (DMF) was added drop-wise to a 10 ml, 0.075% (w/w) poly(allylamine) (PA, $M_w \sim$ 17 000 g/mol) in PBS solution, with continuous stirring at room temperature. Care was taken to avoid precipitation by controlling the rate of NHS-BP addition to the poly(allylamine) (PA) solution. The PA/NHS-BP solution was left to react overnight under subdued lights and then quenched by adding 50 $\mu$l ethanolamine. The resulting product (PA-BP) was purified by dialysis using 3000 molecular weight cut-off dialysis tubing (Spectrum, CA) against PBS. The bath was periodically changed and monitored by UV-vis until there was no appreciable absorbance at 260 nm due to unreacted NHS-BP.

*Oxidation of agarose−OH to agarose−COOH*

The primary hydroxyl groups of agarose (agarose−OH) were oxidized to carboxylic acid groups (agarose−COOH) according to a modified published procedure [22, 23]. Briefly, 1 g of agarose was suspended in 50 ml of water and stirred. Twenty mg of 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) was added to the agarose suspension with 0.1 g of sodium bromide and 2.5 ml (13% w/w) sodium hypochlorite solution. The oxidization reaction was then initiated by adjusting the pH of the suspension to pH 10.8 with 1.0 M NaOH and the pH was maintained by continuously titrating 0.5 M NaOH to the suspension under well-stirred conditions. Since the oxidation quantitatively produced carboxylic acids that needed to be neutralized by NaOH, the extent of the oxidation was followed by the amount of the 0.5 M NaOH required to maintain the solution at pH 10.8 [24]. For example, if 6.4 ml of 0.5 M NaOH were added, it would indicate that the oxidation was complete, i.e. 100% of the primary hydroxyl groups were converted to carboxylic acid groups. Unless otherwise indicated, all agarose−COOH samples had 0.8 ml of 0.5 M NaOH added which translates to 12.5% of the hydroxyl groups oxidized to carboxylic acid groups. The oxidation reaction was conducted at 0°C and the reaction was quenched by the addition of sodium borohydride ($NaBH_4$) at the desired degree of oxidation. The oxidized agarose suspension was washed 5–6 times by centrifugation-washing cycles in deionized distilled water. Subsequently, the oxidized gel was freeze-dried and stored at −20°C in the dark prior to use.

FTIR absorbance spectra (Galaxy Series 5000 spectrometer) were taken of thin agarose films, to confirm the oxidation of agarose−OH to agarose−COOH. Thin agarose−COOH films were prepared from 1% agarose solution, cast onto ZnSe disks and air-dried.

*Coupling of poly(allyl amine)-benzophenone (PA-BP) to agarose−COOH*

PA-BP was coupled to agarose−COOH by EDC activation. Briefly, 0.1 g of agarose−COOH was dissolved in 10 ml of water by microwaving for 45 s and then 0.3904 g (0.002 mol) of 2-(N-morpholino)-ethanesulfonic acid (MES) were added followed by the addition of 15 mg ($7.8 \times 10^{-5}$ mol) of EDC for 5 min. Then 10 ml of 0.075% (w/w) PA-BP in PBS solution were added. The reaction was carried out overnight at 37°C and the products were purified by dialysis (MWCO 50k, Spectrum, CA) against PBS. The resulting photoactive agarose gel is referred to as agarose-BP.

*Determination of photoimmobilization yield of native OVA to agarose-BP*

One ml of a 2% photoactive agarose solution in PBS (agarose-BP) was mixed with 1 ml of I-125 radiolabeled OVA in PBS solution (2 mg/ml) to form 2 ml of a 1% agarose solution. This resulting solution was cast into a 35 mm Petri dish and set at room temperature overnight in a hydrated environment. The resulting 2 mm thick gel disk was then cut into pieces and separately weighed. The agarose-BP/OVA disks (agarose-BP-1) were irradiated at 355 nm for 45 min using a UV reactor equipped with 8 mercury lamps (Rayonet, Branford, CT). These samples were washed with washing buffer (0.1 M sodium bicarbonate and 0.1 M sodium iodide) until the radioactivity of the buffer was less than twice that of the background noise. Two additional agarose-BP/OVA disks served as controls: (1) where an identical disk was not irradiated but was washed, as described above (agarose-BP-2) and (2) where an identical disk was neither irradiated nor washed (agarose-BP-3). Radioactivity in all three disks (i.e. agarose-BP-1, agarose-BP-2 and agarose-PB-3) was measured as before. Comparisons between the weight-normalized radioactivities of the three samples were used to determine both the photoimmobilization yield (agarose-BP-1 vs. agarose-BP-3) and the non-specific binding of OVA to agarose (agarose-BP-2 vs. agarose-BP-3).

## RESULTS

The goal of this study is to demonstrate the feasibility of photo-immobilizing biomolecules within a three-dimensional agarose gel matrix. In order to achieve this goal, two approaches were pursued using the OVA protein as the model biomolecule: photoactive OVA and photoactive agarose. Since we envision creating a protein gradient that is immobilized by photochemistry, we have focused on photoimmobilization yield and not on amount because the latter is proportional to the concentration of protein present [19]. Increasing the % yield is the challenge.

*Photoactive OVA approach*

In the photoactive protein approach, OVA was modified with BP, to form photoactive OVA-BP. OVA-BP was then dispersed in an agarose matrix and photoimmobi-

lized to the matrix in situ. The coupling of the BP to OVA was confirmed by UV-vis spectrophotometry with a significant absorbance at 260 nm. Figure 2 shows the UV-vis spectra of OVA-BP. Spectrum A shows a single broad absorbance peak due to the adjacent peaks at 260 nm of BP and 280 nm of OVA in OVA-BP molecule. No residual NHS-BP was detected in the washing buffer after the dialysis purification process, as evidenced by a lack of absorbance at 260 nm. It is unlikely that the significant absorbance observed at 260 nm of OVA-BP (inside the dialysis tubing) was due to unreacted NHS-BP because the dialysis tubing that was used had a MWCO of 3000 g/mol, unreacted NHS-BP has a molecular weight of 323.2 g/mol and OVA has an average molecular weight of 45 000 g/mol.

As shown in Fig. 2, as the BP moiety was photolysed with increased UV exposure time, the absorbance at 260 nm decreased and reached a plateau value between 45 and 60 min. Thus, 45 min was chosen as the appropriate UV irradiation time in this study and was used in both photoactive OVA and photoactive agarose approaches.

The degree of substitution of OVA by NHS-BP was determined by a fluorescent assay. when fluorescamine reacts with primary amines, it forms stable fluorophores, the fluorescence of which is proportional to the primary amine content [20]. Taking advantage of this reaction, we determined the number of remaining primary amine groups of OVA-BP by reacting it with fluorescamine and compared the fluorescent signal measured to a calibration curve, which was generated in a parallel experiment with native OVA without BP modification, following the identical procedure. Based

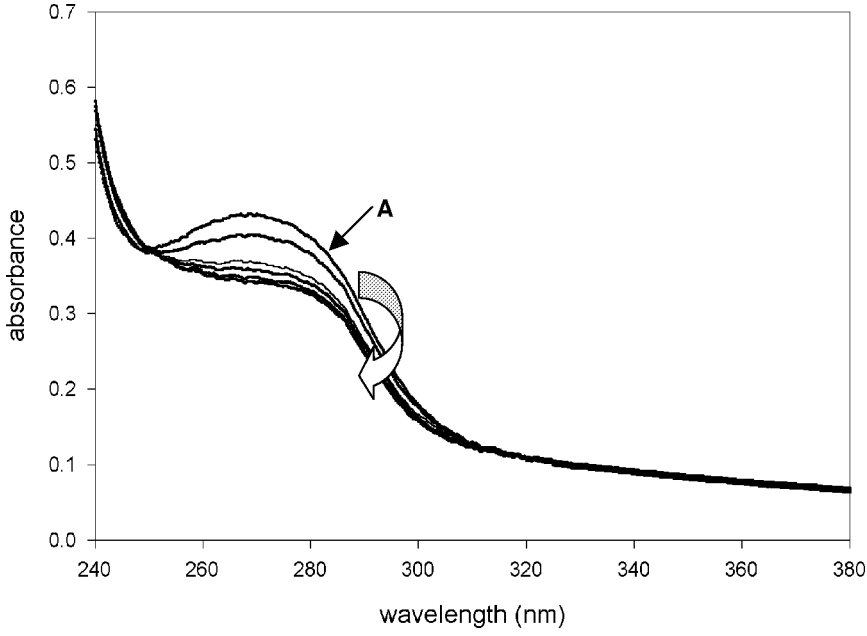
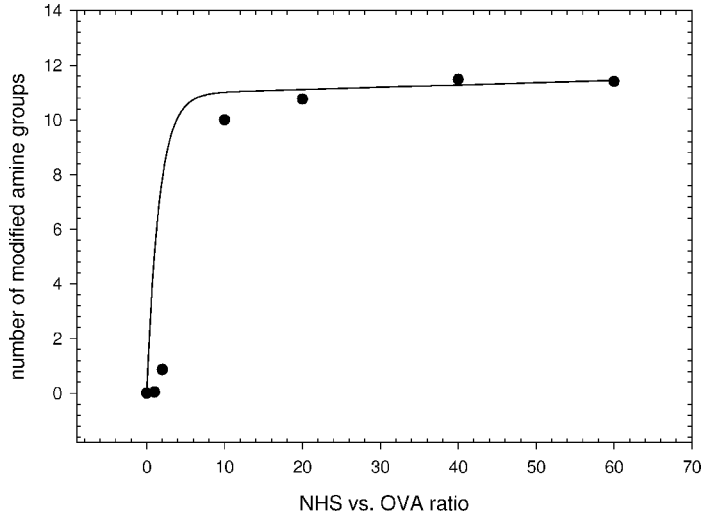
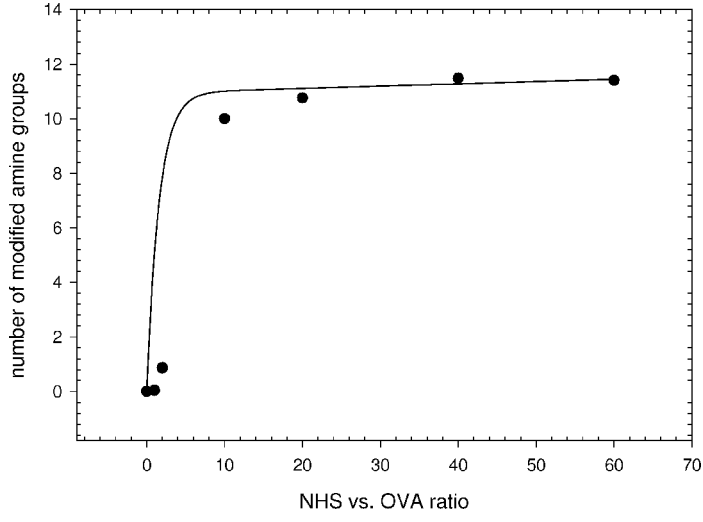



**Figure 2.** UV-vis spectra of OVA-BP were followed to determine the appropriate UV exposure time for photoimmobilization. The exposure times in the direction of the arrow are: 0, 3, 15, 30, 45, 60 min. (A) indicates the OVA-BP without UV irradiation; note the broadened peak.

on the number of primary amine groups of native OVA available for reaction with fluorescamine and the number of amine groups still present on OVA-BP, we were able to calculate the degree of substitution of OVA with BP by subtraction. As shown in Fig. 3, the amount of BP coupled to OVA can be controlled by either the molar ratio between NHS of NHS-BP and OVA (Fig. 3a) or reaction time (Fig. 3b). It is interesting to note that the substitution reactions between NHS-BP and OVA seem to reach a plateau at 14 amine groups per OVA molecule (Fig. 3b). Neither




(a)




(b)

**Figure 3.** The degree of OVA substitution by NHS-BP can be controlled by either (a) the molar ratio of NHS to OVA or (b) reaction time.

an increase in NHS-BP:OVA molar ratio nor an increase in reaction time resulted in more than 14 OVA amines being substituted with NHS-BP. The increasing steric hindrance associated with the increasing bulky hydrophobic BP groups may limit further modification. The photoactive OVA-BP was immobilized to the agarose matrix by UV irradiation at 355 nm for 45 min. Using I-125 labeled OVA-BP, we calculated that only 1.8% of the photoactive OVA originally incorporated into the agarose matrix was immobilized and 0.5% of this could be attributed to the non-specific interaction of OVA with the agarose gel. This translates to that an immobilization amount of $0.4 \times 10^{-6}$ mol of OVA in 1 l of agarose gel.

*Photoactive agarose gel approach*

In the photoactive agarose approach, agarose−OH was oxidized to agarose−COOH and poly(allylamine) was modified with benzophenone (PA-BP). The two, i.e. agarose−COOH and PA-BP, were coupled using EDC and then native OVA was dispersed into the photoactive agarose gel and immobilized by UV irradiation.

The hydroxyl groups of agarose were oxidized to carboxylic acids using the TEMPO radical as a mild oxidizer. The degree of oxidation was determined by the amount of NaOH added to maintain a constant pH 10.8 during the reaction. The oxidation reaction has been shown to proceed at an optimal rate at pH 10.8 [25]. Figure 4 compares the FTIR spectra of different agarose gels at different degrees of oxidation, thereby confirming the success of the TEMPO catalyzed reaction, converting agarose−OH to agarose−COOH. As shown in Fig. 4, the ratio of the carbonyl (C=O) stretch peak at 1650 $cm^{-1}$ to the in-plane bend of the hydroxyl (O−H) peak at 1360 $cm^{-1}$ increased with oxidation. For example, the ratio of the (C=O) peak to the (O−H) peak was (A) 0.67 for un-oxidized agarose; (B) 0.87 for 12.5% oxidized agarose−COOH; and (C) 1.89 for 100% oxidized agarose−COOH. It should be noted that the carbonyl peak at 1650 $cm^{-1}$ overlaps with the scissoring motion of H−O−H, which may result in some artifact in the spectra due to the presence of water in the sample (cf. Fig. 4A).

We found that the physical properties of the agarose gel changed with oxidation. Table 1 correlates the relationship between the degree of modification with the physical appearance of the modified agarose gel and its ability to re-gel. The modified agarose gel showed a significant increase in water solubility, especially at higher degrees of modification and thus a decreased ability to gel by the usual hydrogen bonding mechanism. Inclusion of PA-BP, at the concentrations studied, did not significantly alter the physical properties of the gel further. Our observations agree with those of Bellamkonda *et al.* [4] who showed that gel strength and porosity were not significantly affected by the presence of biomolecules.

Interestingly, the 100% oxidized agarose had crosslinking properties that are similar to those of alginate. For example, the 100% oxidized agarose could be crosslinked with divalent calcium (using 1 nM $CaCl_2$) to form a weak gel.

The amine groups of poly(allylamine) were modified with benzophenone and then dialyzed to remove any unreacted NHS-BP. Little to no absorbance was observed at

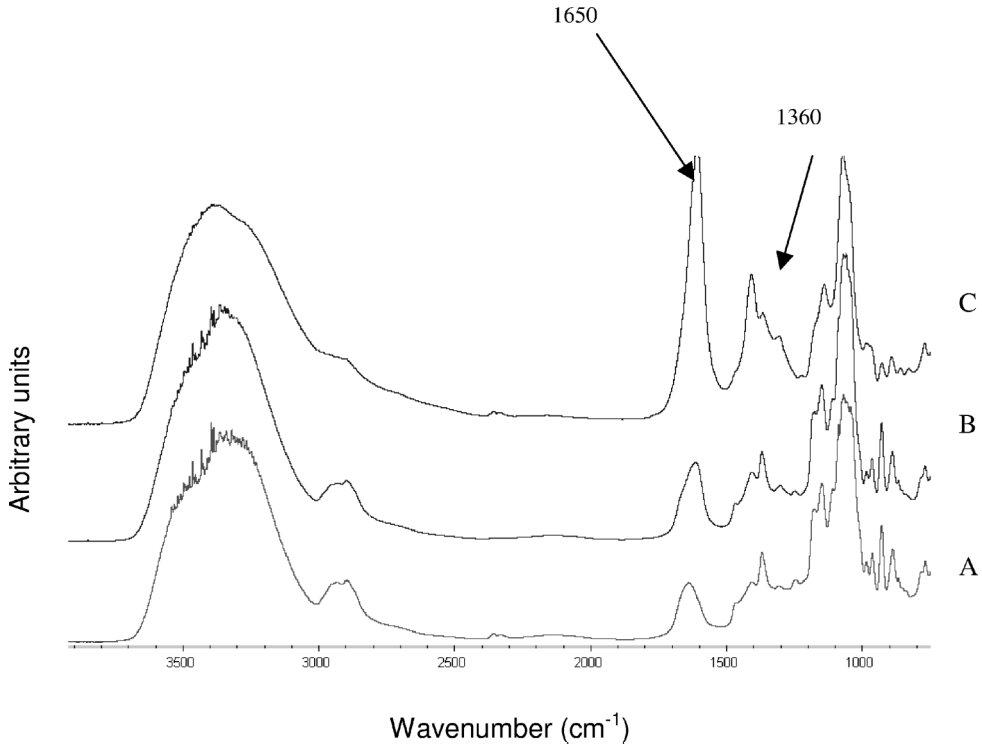



**Figure 4.** Comparison of FTIR spectra of agarose at different degrees of oxidation: (A) native agarose; (B) 12.5% oxidation; (C) 100% oxidation. The peaks at 1650 $cm^{-1}$ and 1360 $cm^{-1}$ are the C=O stretch and O—H in-plane bend, respectively.

**Table 1.**
Correlation of the degree of oxidation, physical appearance and ability to re-gel of oxidized agarose

| Volume of NaOH added (ml)[a] | Theoretical degree of oxidation | Physical appearance after modification | Ability to re-gel after re-solubilization |
|---|---|---|---|
| 6.4 | 100% | transparent liquid | N/A |
| 3.2 | 50% | viscous slag/paste | No[b] |
| 1.6 | 25% | gel | Yes — weak gel |
| 0.8 | 12.5% | gel | Yes — strong gel |

[a] NaOH concentration of 0.5 M.
[b] Only gelled at pH 4.7 and 4°C (overnight).

260 nm (due to the unreacted NHS-BP) in the dialysate washing buffer, indicating quantitative conversion. Assuming 100% conversion, we estimate that 60 BP groups were present per poly(allyamine) which is similar to what has been reported in the literature [26] — i.e. 80 BP molecules per poly(allylamine) molecule. (This result is significantly greater than the maximum of 14 BP molecules incorporated per OVA molecule achieved in the photoactive OVA approach.)

Native OVA was immobilized to the photoactive agarose matrix (agarose-BP) by UV irradiation at 355 nm for 45 min. Using I-125 labeled OVA, we determined that 9.3% of the photoactive OVA originally incorporated into the agarose matrix was immobilized and ~0.5% of this could be attributed to the non-specific interaction of OVA with the agarose gel. This corresponds to $2.1 \times 10^{-6}$ mol of OVA immobilized within 1 l of agarose gel. Thus the photoimmobilization yield increased relative to that which had been previously reported in the literature of 1% [12] to 1.8% for the photoactive OVA approach and to 9.3% for the photoactive agarose approach.

## DISCUSSION

We studied two general photoimmobilization methods, using OVA as the model molecule for its immobilization in a 3D hydrogel matrix. In comparison with other immobilization methods, photoimmobilizatin has the advantage of being simple and versatile to a variety of functional groups (or no functional groups at all). Furthermore, concerns about the bioactivity of UV-irradiated biomolecules have largely subsided after it was shown that the bioactivity is mostly unaffected when molecules are irradiated at wavelengths of 350 nm or longer [27].

Recently, patterned biomolecule surfaces were created by UV irradiation of photoactive-biomolecule conjugates through a photomask [28]. Moreover, agarose has been modified with a tripeptide, arginine-glycine-aspartic acid (RGD), via photo-irradiation [13]. This resulting agarose-RGD matrix elicited significant neurite outgrowth from dorsal root ganglion cells (DRGs) relative to native agarose gel, yet the immobilization yield was only about 1% [12]. We believe that this low yield could be attributed to the limited interactions between the hydrophobic photoactive BP and the hydrophilic environment (1% agarose and 99% water) in which the photoactive moiety was dispersed.

To determine whether we could improve the photoimmobilization yield, we immobilized OVA using either a photoactive OVA or a photoactive agarose. Since effective photoimmobilization can only take place when the activated BP triplet radical is within 3.1 Å of an agarose molecule [29], the interactions between the BP and agarose likely dictate photoimmobilization yield. Thus we explored two ways to improve the interaction between hydrophilic agarose and hydrophobic BP, and thereby increase the photoimmobilization yield.

In the photoactive OVA approach, OVA-BP was mixed with agarose prior to UV-irradiation and resulted in a 1.8% yield. While this yield is slightly greater than that previously achieved [12], the yield is low. We postulate that this low yield may result from the BP moiety being 'buried' within the OVA core and thus unavailable for reaction with agarose.

In the photoactive agarose approach, poly(allylamine) was modified with BP which was subsequently bound to agarose−COOH prior to binding OVA by photo-irradiation. This method alleviates, at least in part, the poor hydrophobic-hydrophilic interaction between BP and agarose. Furthermore, we speculate that

the hydrophobic BP groups can better interact with the amphiphilic protein vs. the hydrophilic agarose by having greater proximity between the photoactive moiety and the protein molecule. This may explain why the greater immobilization yield of 9.3% was achieved using the photoactive agarose approach. Additionally, the use of poly(allylamine) effectively increased the amount of BP incorporated into agarose and this in turn improved the photoimmobilization coupling yield of OVA and agarose.

Although it has been demonstrated that the inclusion of peptides/proteins will not significantly change the porosity and mechanical strength of agarose gel, we found that the oxidation of agarose−OH to agarose−COOH decreased agarose's 'gel−ability' (*cf.* Table 1). This problem could be circumvented by controlling the oxidation to lower degrees. We found that at a 12.5% degree of oxidation, agarose gelled with satisfactory mechanical integrity and thermal reversibility while having sufficient carboxylic acid groups for further modifications.

Photochemistry has been widely used to pattern biomolecules with certain spatial features. For example, Borkenhagan and colleagues [13] photoimmobilized cell adhesive peptides in agarose gel using laser patterning. By varying UV exposure time, a concentration gradient of oligopeptide was immobilized on a 2-dimensional substrate over a 0.3 mm distance [17, 18]. We are also interested in creating a photoimmobilization protein gradient and focused on controlling the interactions between hydrogel matrices, biomolecules and photoactive moieties to improve the photimmobilization yield. Since the amount of protein photo-immobilized is proportional to the amount of protein present [19], it is possible o immobilize a biomolecule concentration gradient in 3-dimensions *in situ*. In ongoing studies, we are investigating methods to optimize the creation of a stable concentration gradient in a 3D hydrogel matrix.

## CONCLUSIONS

We successfully modified agarose with OVA using photoimmobilization technology. We improved the coupling yield to 9.3%, which is a significant improvement over previously published yields of 1%. By using poly(allyamine), we were able to increase the number of BP groups introduced to agarose and available for reaction with OVA. This methodology is versatile to any protein or neurotrophic factor because it is the matrix that is modified, and not the bioactive molecule, prior to photoimmobilization. We are currently exploring methods of immobilizing a concentration gradient of neurotrophic factors for guided axonal regeneration.

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