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Micropreparative Gel Electrophoresis for Purification of Nanoscale Bioconjugates

Sayyed Hashem Sajjadi, Shang-Jung Wu, Yahya Rabbani, Vitalijs Zubkovs, Hossein Ahmadzadeh,* Elaheh K. Goharshadi, and Ardemis A. Boghossian*

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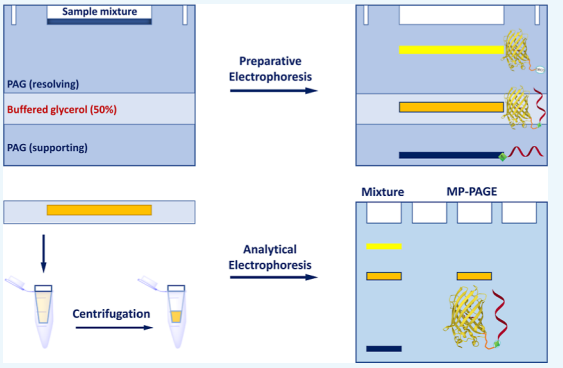
ABSTRACT: Conventional techniques for purifying macromolecular conjugates often require complex and costly installments that are inaccessible to most laboratories. In this work, we develop a one-step micropreparative method based on a trilayered polyacrylamide gel electrophoresis (MP-PAGE) setup to purify biological samples, synthetic nanoparticles, as well as biohybrid complexes. We apply this method to recover DNA from a ladder mixture with yields of up to 90%, compared to the 58% yield obtained using the conventional crush-and-soak method. MP-PAGE was also able to isolate enhanced yellow fluorescence protein (EYFP) from crude cell extract with 90% purity, which is comparable to purities achieved through a more complex two-step purification procedure involving size exclusion and immobilized metal-ion affinity chromatography. This technique was further extended to demonstrate size-dependent separation of a commercial mixture of graphene quantum dots (GQDs) into three different fractions with distinct optical properties. Finally, MP-PAGE was used to isolate DNA−EYFP and DNA−GQD bioconjugates from their reaction mixture of DNA and EYFP and GQD precursors, samples that otherwise could not be effectively purified by conventional chromatography. MP-PAGE thus offers a rapid and versatile means of purifying biological and synthetic nanomaterials without the need for specialized equipment.



INTRODUCTION

The purification of biological macromolecules is the crux of many bioanalytic and biotechnological advances.[1] Macromolecules like proteins and nucleic acids, as well as organic/inorganic nanoparticles, can be purified using a slew of techniques including high-speed centrifugation, membrane-based ultrafiltration, precipitation, electrophoresis, and chromatography.[2−5] These techniques differ in aspects such as scalability, throughput, yield, purity, precision, laboratory accessibility, and procedural complexity. These aspects determine the technique (or combination of techniques) that is most suitable for isolating a particular macromolecule for a given application.

Although purification methods based on flow chromatography benefit from relatively high throughput and scalability,[4,6,7] analytical applications, such as sequencing and protein crystallography, require only a small amount of purified protein. For such applications, techniques like polyacrylamide gel electrophoresis (PAGE) are preferred as they can separate biomolecules based on their size and net electric charge.[8−10] PAGE allows for precise control of the pore size in the polyacrylamide gel (PAG), eliminating the need for specialized size-specific chromatography columns. It is capable of separating DNA with a single base-pair resolution[11,12] as well as proteins either in their native or denatured forms.[8] The versatility, high resolution, low cost, and speed of PAGE have made it a standard practice in most biological laboratories.

Although PAGE is typically used for analytical protein separation, several studies have investigated its use as a preparative procedure for purifying proteins.[13−22] Large-scale (milligrams to grams of protein) electrophoresis, for example, can be achieved using preparative columns. However, this setup often generates heat, leading to band broadening and protein denaturation.[8] For this reason, this technique relies on expensive and specialized preparative equipment with cooling systems. These separations are usually followed by post-elution methods for extracting the proteins from the PAG after separation.[23] Depending on the post-elution method, these extractions may require additional specialized setups that can further alter protein activity and/or folding. For example, Hao et al.[14] developed a microscale preparative electrophoresis system for proteins that required distinct separation and elution apparatuses. Other examples of preparative electro-

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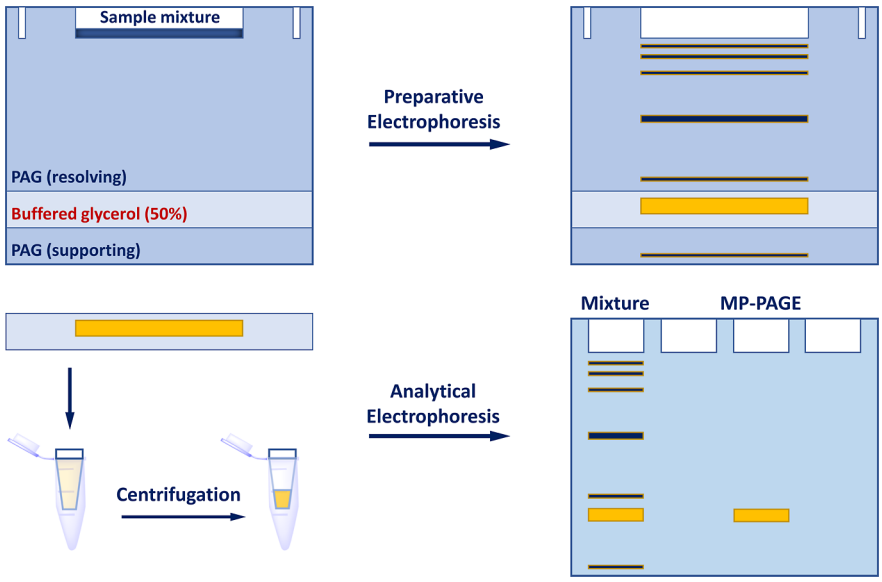

Figure 1. Schematic drawing of MP-PAGE for separating and extracting nanoscale materials. The top-left schematic shows the composition of an MP-PAGE gel containing a top resolving PAG, a glycerol layer for sample extraction, and a bottom supporting gel. The resolving gel is used at an adequate gel composition and run at appropriate voltages for biomolecule/nanoparticle separation. The band containing the desired biomolecule of interest (yellow band in the top-right) is selectively eluted into the glycerol layer and run through a centrifugal filter (bottom-left) to remove the glycerol. An analytical gel is run on the extract (bottom-right) to confirm sample purity relative to the initial mixture.

phoresis approaches require specialized membranes. In one such case, Fadouloglou[15] used a membrane in the electrophoresis setup to separate DNA. The dedicated effort for customizing such setups, however, limits the accessibility of the technology to specialized laboratories.

Furthermore, the few preparative gel electrophoresis approaches developed to date have been largely limited to DNA or proteins. These methods are complex and result in significant sample loss, making them unsuitable for dilute and low-yield samples, such as certain DNA−protein bioconjugates. For this reason, the primary preparative approach for bioconjugate purification, the crush-and-soak method,[24] is limited to relatively concentrated reaction samples. Consequently, the purification of such dilute complexes has traditionally been achieved with chromatography or multistep methods that involve tagging biomolecules.[25] For example, Zhou et al.[26] purified DNA−protein conjugates through a multi-step procedure following DNA modification and magnetic bead binding and release steps. This procedure resulted in the extraction of a modified conjugate containing a desthiobiotinylated tag. Yang et al.,[27] on the other hand, developed a sophisticated membrane-based approach for biomolecule isolation, though the extracted biomolecule was accompanied by several impurities.

In this study, we introduce a single-step micropreparative (MP)-PAGE technique for purifying biomolecules, synthetic nanoparticles, and bioconjugates without requiring membranes or chemical modification. The technique utilizes a trilayer slab gel arrangement, as illustrated in Figure 1, which can be easily implemented using a standard vertical electrophoresis setup available in most bioanalysis laboratories. To evaluate the effectiveness of this approach, we compare it with established techniques based on the crush-and-soak method, size exclusion chromatography (SEC), and immobilized metal-ion affinity chromatography (IMAC). The approach is applied to a variety of samples including DNA, a model recombinant enhanced yellow fluorescent protein (EYFP), synthetic graphene quantum dots (GQDs), and DNA−protein and DNA−GQD bioconjugates. These applications demonstrate the success of this approach for purifying dilute, low-yield bioconjugates that are otherwise intractable with existing gel electrophoresis techniques.

RESULTS AND DISCUSSION

Separation of Double-Stranded DNA (ds-DNA). We applied the MP-PAGE setup shown in Figure 1 (and Figures S1 and S2) to separate and extract individual ds-DNA bands from a ladder mixture. In this setup, the desired bands were eluted and collected from the glycerol layer. Compared to buffered aqueous solutions commonly used in specialized electrophoresis flow systems,[15,23] the higher viscosity of the glycerol layer effectively hampers band mobility, allowing distinct DNA bands to remain separated within the glycerol layer. Furthermore, the denser glycerol layer shows limited diffusion into the PAG presolution, enabling solidification of the presolution in the presence of the neighboring glycerol layer. This arrangement was used to separate a 25-bp band from a 12-strand ds-DNA mixture (Figures 2A and S3). The 50 and 75-bp DNA strands were also separated using the same gel composition and operating voltage conditions, demonstrating the versatility of this technique in separating different DNA sizes within a mixture. The calculated recovery yields are 90, 80, and 77% for the 25, 50, and 75-bp DNA strands, respectively (Figure 2B). The gradual decrease in the yields with increasing DNA length is attributed to the resolving gel conditions being optimized to favor separation of the 25-bp strand. The same DNA strands were also separated using the traditional crush-and-soak method with calculated recovery yields of 58, 54, and 24% for the 25, 50, and 75-bp DNA bands, respectively. Compared with MP-PAGE, the crush-and-soak method shows lower extraction yields and an even greater decrease in the extraction of larger DNA strands under the tested conditions. This difference in DNA extraction is largely

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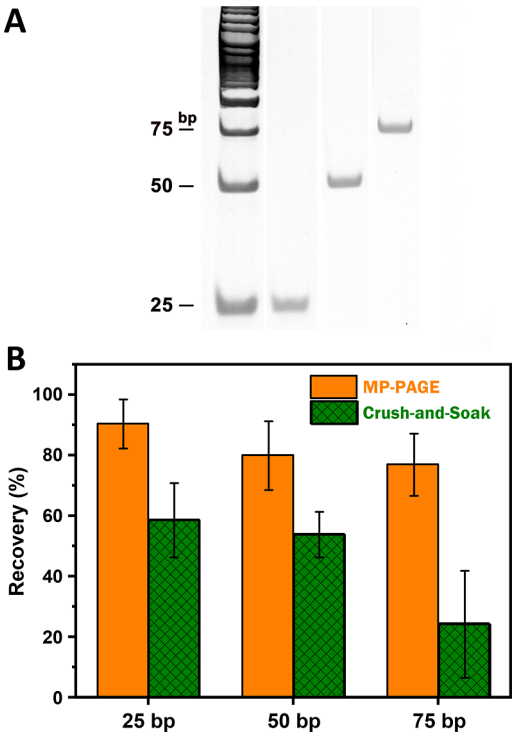

Figure 2. Separation of ds-DNA lengths from a ladder mixture containing 12 different DNA lengths. (A) 12% Native PAGE profiles (stained with SYBR Green, SG) of the MP-PAGE-separated DNA samples. (B) Comparison of the purified DNA recovery yields from the MP-PAGE and the crush-and-soak techniques. Experiments were performed in triplicate, and the error bars represent standard deviations.

attributed to the diffusion-limited separation of the DNA from the solid gel matrix in the crush-and-soak extraction.[28]

Protein Purification: Separation of EYFP from *E. coli* Extracts. The MP-PAGE was also used to recover overexpressed EYFP from recombinant *Escherichia coli*. Figures 3A and S4 show the analytical reducing SDS−PAGE gel of the crude bacterial extract enriched with the recombinant EYFP, accompanied by several contaminating proteins ranging between 10 and 100 kDa. The EYFP is represented by a double band at around the expected size of 27 kDa, which appears under both denaturing and native PAGE conditions. This double band is attributed to the cleavage of a signaling sequence, consistent with previous observations in mammalian[29] as well as *E. coli* expression systems.[30] This hypothesis is further supported by the C-terminal and N-terminal cysteine modifications explored previously in *E. coli*, which showed preferential reactivity for C-terminal modifications that are less likely lost from cleavage.[31] The proteins contained by the bands were extracted and purified using MP-PAGE, SEC, IMAC, as well as a double-extraction method based on IMAC extraction followed by SEC (chromatograms and corresponding Coomassie brilliant blue (CB)-stained gels shown in Figures S5 and S6). Compared with the MP-PAGE sample, which shows only sparse and faint contamination, the SEC sample shows a greater amount of both smaller and larger protein impurities. The impurities are particularly pronounced for sizes around the desired ∼27 kDa band. In contrast, the IMAC extraction shows fewer impurities, with a pronounced contaminating protein band at around 10 kDa. This impurity is attributed to nonspecific binding of histidine- or arginine-rich

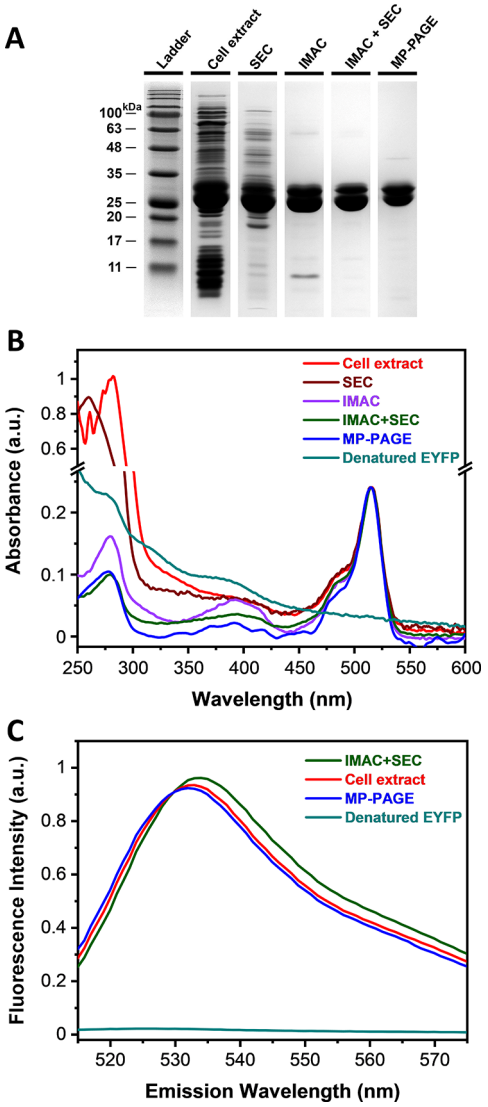

Figure 3. Analysis of protein-containing samples using gel electrophoresis, absorbance, and fluorescence spectroscopy. (A) CB-stained reducing SDS−PAGE profiles of crude cell extract and purified EYFP samples from SEC, IMAC, IMAC + SEC, and MP-PAGE. To compare the impurities for an equivalent amount of EYFP, each lane was loaded with sample volumes that have the same EYFP peak absorption at 514 nm ($A_{514\ nm}$ = 0.2). (B) UV−visible spectra of crude cell extract, purified EYFP samples from SEC, IMAC, IMAC + SEC, and MP-PAGE, as well as denatured EYFP. Denatured EYFP corresponds to the IMAC + SEC sample heated to 95 °C for 5 min. (C) Fluorescence spectra of crude cell extract and purified EYFP samples from IMAC + SEC (as the purest protein sample) and MP-PAGE, as well as denatured EYFP described in (B). The samples were excited at 490 nm, and fluorescence spectra were normalized by the EYFP concentration.

proteins that can intrinsically bind to the nickel column. Compared with SEC and IMAC alone, the double-extraction IMAC + SEC method shows the least contamination, showing only faint impurity bands. Based on the gel analysis, the sample purity is comparable to that achieved with MP-PAGE, except for a faint impurity band at 40 kDa that is more pronounced in the MP-PAGE sample. Because of the comparable purity, the samples purified from the IMAC + SEC method were used for further comparison with MP-PAGE.

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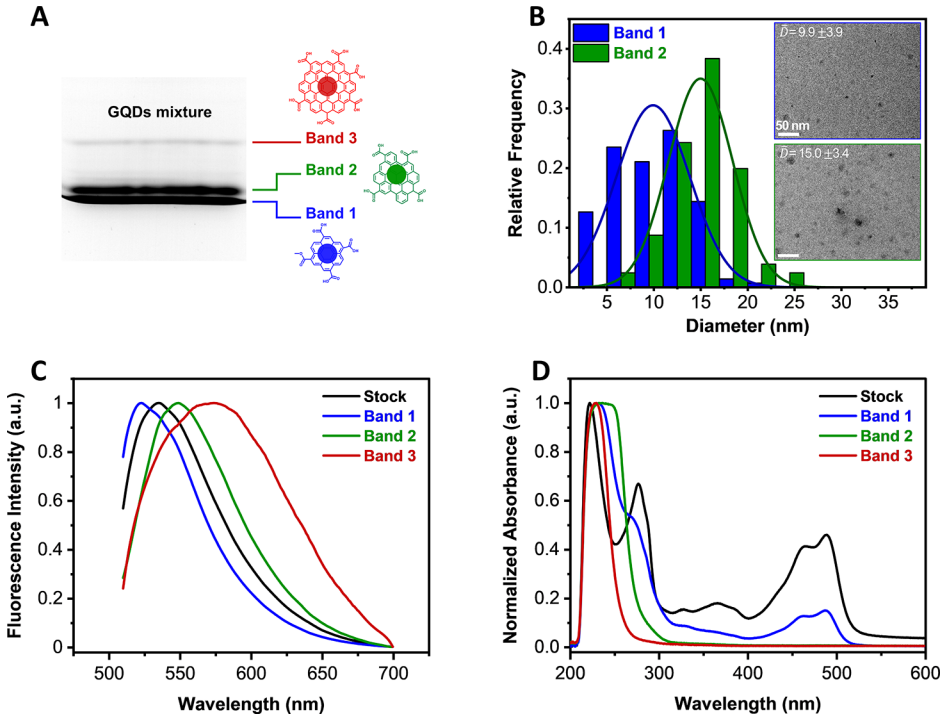

Figure 4. Separation of GQDs of different sizes. (A) PAGE profiles of commercial GQDs. The separated bands are visible under blue light. (B) Analysis of size distribution using TEM for band 1 and band 2. The inset shows representative TEM images of the separated bands. The frequency size distribution is based on measurements from 200 particles. (C) Fluorescence (λ_{ex} = 485 nm) and (D) UV−visible spectra of the commercial stock and the separated fractions shown in (A).

The purity and integrity of the samples were further analyzed with absorption and fluorescence spectroscopy. All extracted samples, except the denatured EYFP, show an absorption peak at 514 nm (Figure 3B). Furthermore, Figure 3C shows the fluorescence of EYFP with its characteristic peak at around 533 nm upon 490 nm excitation. Both the absorption and fluorescence peaks were diminished when the samples were heated to 95 °C for 5 min. Because this treatment denatures EYFP, the diminished absorption and fluorescence peaks verify that the detected optical signatures correspond to a protein that is properly folded. The absorption spectra were also used to calculate the sample purity. Assuming a relatively pure protein solution from the IMAC−SEC sample, we calculated an effective EYFP extinction coefficient of $\varepsilon_{EYFP,280} \approx 1.23$ mL mg^{-1} cm^{-1} based on the total protein concentration (as determined by the bicinchoninic acid assay, BCA) of the IMAC−SEC sample and the optical absorption measured at 280 nm ($A_{sample,280}$), which corresponds to the nonspecific absorption peak of proteins containing aromatic residues. The total native EYFP protein concentration was calculated based on its characteristic absorption peak at 514 nm ($A_{sample,514}$) with an extinction coefficient, $\varepsilon_{EYFP,514}$, of 2.98 mL mg^{-1} cm^{-1}.[64] By comparing the calculated protein concentration at 514 nm with the effective concentration at 280 nm, the native-EYFP purity (% P) in the different samples was calculated using the following equation

$$\% P = \frac{[A_{sample,514}/\varepsilon_{EYFP,514}]}{[A_{sample,280}/\varepsilon_{EYFP,280}]} \quad (1)$$

Based on this calculation, the protein purity is found to be 16, 58, 94, and 90% for SEC, IMAC, IMAC + SEC, and MP-PAGE, respectively.

Purification of GQDs. The ability to fractionate low-yield and dilute samples is especially useful for costly or difficult-to-scale synthetic nanomaterials like GQDs. GQDs are available as commercial mixtures with photoluminescence properties that vary with size, shape, functionality, as well as the type and extent of defects.[32,33] Compared to conventional reporters such as fluorescent proteins, inorganic quantum dots, and organic dyes,[34] GQDs benefit from both enhanced photostability and tunable photoluminescence wavelengths. The ability to readily fractionate GQDs of different wavelengths would therefore promote high-efficiency and multimodal optical technologies based on these nanomaterials.[35−40] Although techniques such as density gradient centrifugation are common for separating such nanomaterials, these techniques often suffer from significant losses and tedious preparation protocols.[41]

We therefore demonstrated MP-PAGE on the separation of GQDs. Because the GQDs contain several functional groups, tris-glycine (TG) buffer (pH 8.43) was used to deprotonate the carboxylic groups on the surface of the GQDs and enable electrophoretic mobility.[5] The commercially available GQD mixture contains three visible fluorescence bands that can be tracked in PAG under illumination with blue light (Figure 4A). Transmission electron microscopy (TEM) analysis (Figure 4B) shows that bands 1 and 2 comprise nanoparticles with average diameters of 9.9 and 15.0 nm, respectively. Under 485 nm excitation, the mixture demonstrates peak fluorescence emissions around 535 nm, falling within the range of peaks observed from the isolated bands at 524, 549, and 575 nm for bands 1, 2, and 3, respectively (Figure 4C). A closer examination, as illustrated in Figure 4A,B, reveals a consistent red shift in the fluorescence emissions of the bands, corresponding to a decrease in mobilities and an increase in

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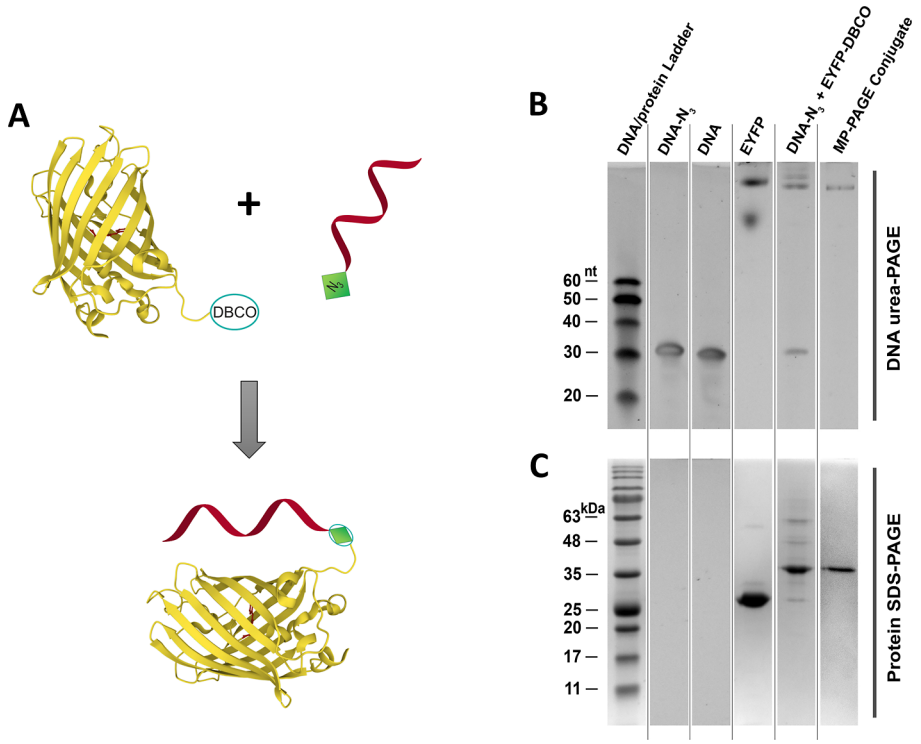

Figure 5. Purification of DNA–EYFP bioconjugate. (A) Conjugation of DNA and EYFP through click chemistry. (B) Urea–PAGE profiles showing DNA, EYFP, and conjugate bands. (C) Reducing SDS–PAGE profiles showing EYFP and conjugate bands. DNA–N_3 + EYFP–DBCO corresponds to the reaction sample after 4 h of incubation followed by filtration using a 30 kDa Amicon centrifugal device.

size. This observation aligns with previous studies predicting a red-shift in emission fluorescence with increasing size.[34,42,43] Therefore, these findings illustrate that the MP-PAGE can effectively segregate GQDs of different sizes within each band. We note, however, an overall stronger signal for bands 1 and 2 in the gel (Figure 4A) as well as a closer proximity of the mixture's peak fluorescence to those of bands 1 and 2 compared with that of band 3 (Figure 4C). Although such findings could suggest higher concentrations of GQDs from bands 1 and 2 in the commercial mixture, these observations may also stem from differences in the quantum yields of the three bands.

The bands were therefore further analyzed with UV–visible absorption spectroscopy. As shown in Figure 4D, band 3 shows the narrowest absorption features, with a peak absorption at 229 nm. Band 1, on the other hand, shows the broadest absorption range with peaks at 268, 460, and 488 nm. Compared to the separated bands, the commercial mixture contains peaks, such as those at 277, 325, and 365 nm, in addition to those observed in the three separated bands. The additional absorption peaks in the mixture could indicate the presence of moieties that are nonfluorescent under the conditions used to track and separate the bands. Nonetheless, the distinct absorption spectra of these three bands confirm the fractionation of GQDs with different optoelectronic properties.

Purification of Nanoscale Bioconjugates. Bioconjugates are used in a variety of applications including nanofabrication, target recognition, signal amplification, catalysis, chemical modification, and immunology.[26,44,45] Several approaches have been developed to conjugate proteins or nanoparticles with DNA.[46−52] However, the conjugation product often requires extensive treatment to remove the unreacted precursors. Methods such as crush-and-soak, which is used for the DNA recovery described above, result in poor yields.[24,53] Alternative methods, such as the biotin displacement strategy developed by Zhou et al.,[26] ion-exchange chromatography,[54,55] SEC,[56] and using Ni^{2+}-NTA magnetic beads,[57] can achieve higher yields. However, the extracted conjugates are often accompanied by sample impurities, such as unreacted reagents. These methods also rely on specialized equipment and columns.

In our study, we aim to purify a DNA–EYFP bioconjugate synthesized via azide–DBCO click chemistry[31] (Figure 5A). The single-stranded DNA (ss-DNA)–azide was mixed with DBCO–EYFP to form a covalently linked ss-DNA–EYFP based on stable triazole formation. The successful conjugation of the ss-DNA–EYFP was confirmed with urea–PAGE, which shows the formation of a new conjugate band following the reaction (Figures 5B and S7). The conjugated sample shows a decrease in electrophoretic mobility compared to the non-conjugated EYFP. This decrease is attributed to the negatively charged oligonucleotide that is appended to the modified protein.[58] The conjugation was also confirmed with SDS–PAGE (Figure 5C), which shows an ∼9 kDa increase in molecular weight. This difference corresponds to the attachment of one oligonucleotide per EYFP. By contrast, the electrophoretic mobilities of DNA (Figure 5B) and protein (Figure 5C) do not change in the absence of azide and DBCO. These results suggest that the new bands appearing in the samples indeed originate from the covalent conjugation of EYFP to DNA.

In addition to verifying the bioconjugation, urea–PAGE and SDS–PAGE were also used to validate the purity of the MP-PAGE extraction. The limited amount of sample could not be purified with SEC due to chromatography losses and the inability of SEC to discern unreacted proteins from their

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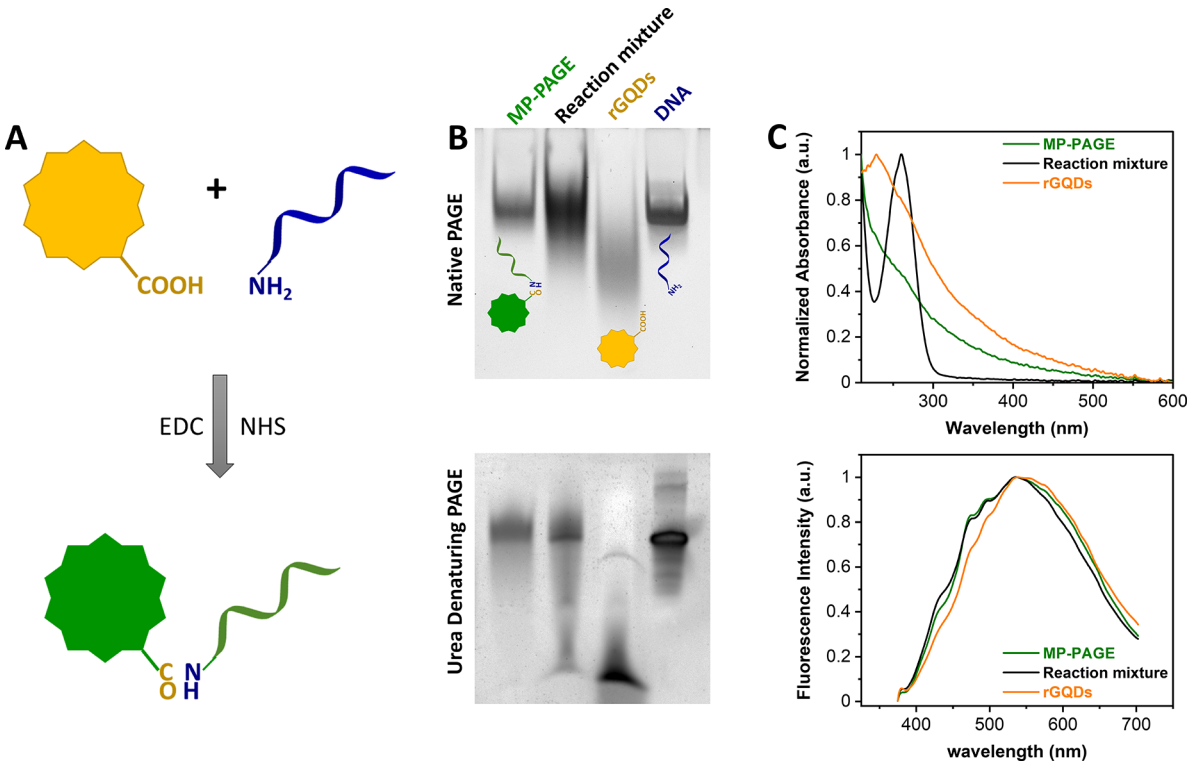

Figure 6. Purification of DNA−GQD bioconjugate. (A) Conjugation of DNA and GQDs through carbodiimide cross-linking. (B) Native and urea denaturing PAGE showing SG stain of MP-PAGE-purified bioconjugate, the unpurified reaction mixture, rGQDs, and DNA. (C) UV−vis absorbance and fluorescence spectra (λ_{ex} = 350 nm) of the rGQDs, the unpurified reaction mixture, and the MP-PAGE-purified bioconjugate.

conjugates. However, a limited amount was sufficient for MP-PAGE purification. Furthermore, the purified MP-PAGE sample shows complete isolation of the conjugate from the unreacted compounds (Figure 5B,C). By contrast, unreacted DNA and proteins appear in the nonpurified samples, even after filtration through a 30 kDa centrifugal membrane (Figures 5B and S7).

In addition to DNA−EYFP, the MP-PAGE technique was used to isolate DNA−GQD bioconjugates. The DNA−GQD bioconjugates were synthesized through carbodiimide cross-linker chemistry[59] (Figure 6A). A 15% native gel was used to separate the conjugate band from the components of the reaction mixture containing DNA and reduced GQDs (rGQDs). The effectiveness of MP-PAGE purification in removing unreacted rGQDs was confirmed through native and urea denaturing PAGE (Figure 6B). However, the native and urea denaturing PAGE results did not distinguish the mobility difference between the DNA−GQD and the free DNA precursor, as the GQDs are much smaller in size. Nonetheless, the PAGE results indicate the presence of impurities in the reaction mixture, which are absent in the MP-PAGE-purified conjugates. These findings are consistent with the UV−vis absorbance and fluorescence measurements that additionally verify the isolation of the bioconjugate (Figure 6C). The top panel of Figure 6C shows a sharp absorption peak at 260 nm in the reaction mixture, corresponding to excess unreacted DNA. This characteristic peak appears as a shoulder in the spectrum of the MP-PAGE-purified sample, suggesting the removal of excess DNA. The conjugation of the remaining DNA to the rGQDs was further confirmed through fluorescence spectroscopy. The bottom panel of Figure 6C illustrates an overlapping peak between the MP-PAGE-purified sample and rGQDs, thereby confirming the presence of GQDs in the MP-PAGE bioconjugate.

OUTLOOK AND CONCLUSIONS

Previous studies have explored the use of gel electrophoresis for the preparative purification of proteins either in column or slab formats.[14,17,19,60,61] These studies relied on either continuous or stopped-flow elution during electrophoresis, or they required subsequent gel slicing and extraction steps that limit their yield.[23] Although a variety of such apparatuses have become available commercially, they are complex, expensive, or difficult to set up.[16] The MP-PAGE approach presented herein uses standard electrophoretic equipment that is readily available in most bioanalysis laboratories to purify proteins, nucleic acids, and bioconjugates. Unlike other preparative gel electrophoresis methods that require high elution volumes and yield diluted protein extracts, MP-PAGE samples are eluted in a small volume (1−2 mL) of buffer that can be collected with a syringe. The MP-PAGE setup also obviates the need for complex preparation and shutdown procedures as well as specialized equipment, such as elution chambers, external pumps, and external fraction collectors.

The versatility of this MP-PAGE method was demonstrated by purifying four distinct samples: a nucleic acid, a protein, a bioconjugate, and a synthetic nanoparticle sample in multicomponent mixtures. The bioconjugate sample used in this study could not be purified using existing techniques due to the low sample amount. Although all the samples used in this work relied on fluorescence to allow us to visually track the samples in the gel, the technique is applicable to nonfluorescent samples as well. For example, protein samples could be prestained with dyes such as Coomassie G-250[16] and

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Instant Band (EZBiolab) and run in parallel to the MP-PAGE to track the appropriate collection time.

The MP-PAGE method is particularly convenient for quick, cost-effective, and small-volume protein purification in research laboratories. The protein purity achieved with MP-PAGE is comparable to that obtained using chromatography techniques, which are considered the gold standard for protein purification. In this study, MP-PAGE purification was performed within 2 h compared to the two-step IMAC + SEC purification that required nearly 1 day to achieve comparable purities. The ability to tailor the separation resolution by altering the resolving gel concentration further provides a low-cost, tunable approach for purifying proteins of various sizes and charges. In contrast, chromatography techniques require different columns for proteins of different sizes, charges, and metal-ion affinities. Compared to IMAC, MP-PAGE also does not require a protein tag for purification, circumventing the need for further protein engineering and overcoming losses from subsequent detagging steps. Furthermore, previous studies have shown that native PAGE can be used to separate and purify proteins from intact *E. coli* without disrupting the cells.[17] This ability would allow MP-PAGE to separate proteins in the absence of preliminary cell disruption steps that are necessary for chromatography techniques and can contribute to additional protein loss. We note, however, that these advantages are largely limited to applications that require small protein amounts. In such cases, chromatography and alternative flow-through systems remain beneficial for a larger volume protein purification.

MP-PAGE can also be extended to separate other biomolecules, such as DNA, DNA−protein conjugates, and nanoparticles. The MP-PAGE separation demonstrated in this study, for example, was performed under native conditions based on differences in both protein charge and size. MP-PAGE can also be performed in SDS-denaturing conditions, which can separate proteins only based on size. However, though SDS separation benefits from improved separation resolution compared to continuous native-PAGE, this application is limited to proteins that can be refolded. In addition, this platform can also be applied to a diverse range of nanoscale materials, including oligonucleotide origami and nanoparticle conjugates. Subsequent buffer exchange and centrifugal filtration may limit the recovery of samples like DNA origami that can be more effectively recovered through alternative techniques, such as poly(ethylene glycol) (PEG) precipitation. However, such precipitation approaches are limited to separating DNA macrostructures only from much smaller oligonucleotides; MP-PAGE enables the separation of DNA macrostructures from larger incomplete structures. As is the case with proteins and DNA, separation of these conjugates can be achieved by varying the concentration, height, and thickness of the resolving gel, in addition to buffer composition and applied potential in the electrophoresis device. The use of alternative gel systems, such as discontinuous pH gradient or PAG concentration gradient gels, could also be implemented to further optimize separation.

MATERIALS AND METHODS

Materials. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, ABCR), dibenzocyclooctyne-maleimide (DBCO, Tokyo Chemical Industry), GQD solution (Sigma), 1-ethyl-3-(3-dimenthylaminopropyl)-carbodiimide (EDC, Sigma), and *N*-hydroxysulfosuccinimide sodium salt (Sulfo-NHS, Sigma) were used without further purification. Acrylamide/bis-(acrylamide) stock solutions, 19:1 and 37.5:1, were purchased from Carl Roth. The ss-DNA sequences used in this study were provided by Microsynth.

Preparation of EYFP Enriched Protein Extract. Competent *E. coli* BL21 cells harboring the recombinant plasmid encoding EYFP were cultured in a 2×YT medium supplemented with carbenicillin antibiotics. The cell culture was harvested and lysed for protein extraction. The insoluble materials were pelleted by centrifugation, and the supernatant (cell extract) was filtered through a 0.2 μm porous sterile filter and stored at 4 °C.

EYFP Purification Using Chromatography Techniques: IMAC and SEC. IMAC purification was performed using a His-Trap HP 1 mL (GE Healthcare) on an ÄKTA START protein purification system (GE Healthcare). The sample was loaded in a buffer containing 20 mM phosphate buffer saline (PBS, pH 7.4), 500 mM NaCl, and 20 mM imidazole and then eluted in 20 mM PBS containing 500 mM NaCl and 500 mM imidazole. 15.0 mL of the cell extract was loaded into the column, and the chromatography was carried out at a flow rate of 1.0 mL/min for all steps (loading, washing, and His-tagged protein elution).

The SEC purification was achieved using a HiPrep 16/60 Sephacryl S-300 high resolution column (GE Healthcare) on the same ÄKTA system. The elution buffer contained 10 mM PBS (pH 7.4) and 140 mM NaCl. 3.0 mL of the sample was loaded into the column. The chromatography was carried out for 4 h with a flow rate of 0.5 mL/min. The sample mixture was separated and eluted into more than 40 fractions. The eluted fractions containing purified protein samples were concentrated using a 10 kDa Amicon centrifugal filter (Merck Millipore) and the elution buffer suspending the purified protein was replaced with EDTA (10 mM)−PBS (1×) buffer (pH 7.0) for long-term storage.

ss-DNA Conjugation to EYFP or GQD. ss-DNA−EYFP bioconjugation was performed as described previously.[31] The purified EYFP was reduced using 10 mM TCEP in EDTA−PBS buffer for 1 h while being shaken at 500 rpm in an Eppendorf thermomixer at 5 °C. The product was purified using a PD midiTrap G-25 desalting column (GE Healthcare) eluted with the same buffer. Freshly prepared 20 mM DBCO in dimethyl sulfoxide was mixed with reduced EYFP (0.05 mM). The sample was shaken overnight at 5 °C, and the unreacted DBCO was subsequently removed using a PD midiTrap G-25 desalting column. EYFP−DBCO was mixed with 5′-N_3-GCA TGA ACT AAC GGA TCC CCT ATC AGG ACG-3′ (BH-N_3) in a 1:4 molar ratio. The sample was left to react at room temperature, accompanied by shaking for 4 h. A mixture of EYFP (without DBCO) and BH (without an azide group) was incubated in the same manner as the control.

The ss-DNA was conjugated to GQDs according to a procedure reported by Qian et al.[59] Briefly, rGQDs, synthesized through oxidation/scission of commercial reduced graphene oxide,[62] were dissolved in 4.0 mL of phosphate buffer ([rGQDs] = 1 mg/mL), and the pH of the solution was adjusted to pH 5 to protonate the carboxyl groups of the rGQDs. After the pH was adjusted, EDC (80.0 mg) and sulfo-NHS (80.0 mg) were added to activate the surface carboxylic group for 30 min at room temperature with continuous stirring. The pH of solution was then raised to pH 8 to activate the carbodiimide ester for esterification, and an amino-modified DNA solution (5′-NH_2-TTGGTGAAGC-

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TAACGTTGAGG-3′, 15.0 μL, 100.0 μM) was immediately added and allowed to react for 24 h at room temperature with continuous stirring. Finally, the mixture was centrifuged to remove insoluble particles.

MP-PAGE Purification of Nanoscale Materials. An acrylamide/bis(acrylamide) stock solution of 40% (19:1) was used to prepare PAGs in both preparative and analytical DNA-PAGE experiments, while a 30% (37.5:1) solution was used for the protein-PAGE experiments.

The concentration of the PAG could be varied between 4 and 20%, depending on the size of the biomolecule or nanomaterial of interest. The gel contained a 50% glycerol layer in running buffer for collecting the target band, followed by a supporting gel layer in running buffer (Figures 1 and S1). After sample loading, electrophoresis was run until the band of the target molecule reached the glycerol layer. Prior to its elution into the glycerol layer, this layer and the supporting gel layer were replaced with new layers to remove undesired species with higher mobilities than the biomolecule of interest (Figure S2). The electrophoresis was reapplied until the band of interest was eluted into a new glycerol layer. The eluted sample was then recovered with a syringe and further concentrated using centrifugal filters for protein and conjugate samples and ethanol precipitation for the DNA sample.

DNA Separation. A ds-DNA ladder (HyperLadder 25−500 bp, Bioline) was prestained with SG and incubated in sample buffer (50% glycerol with 2× tris-boric acid−EDTA, TBE) for 30 min before injection into the gel. The prestaining allowed us to track the DNA bands during electrophoresis. The preparative separation of DNA was performed on a MP-PAGE gel consisting of a 3 cm 12% resolving PAG and 2 cm 50% glycerol layer with 2× TBE buffer. After extraction of the glycerol layer, the separated DNA sample was concentrated by ethanol precipitation, which also removed the tracking dye. For a comparison, the same bands were also separated using a conventional post-elution extraction procedure known as crush-and-soak.[63] For this procedure, the DNA mixture was run in a normal native PAG with the same concentration as the resolving gel in the MP-PAGE setup. The bands of interest were cut after electrophoresis using a scalpel, excised with a needle into small pieces, and soaked in buffer (1× TBE). The sample was incubated overnight, and the DNA that diffused out of the gel was concentrated by ethanol precipitation. The purity and recovery yield of the separated samples were compared using analytical electrophoresis on a 12% resolving PAG. To estimate the recovery yield, the corresponding volumes of the concentrated sample (taking into account the dilution factor for the recovered sample during ethanol precipitation) and the original mixture were run on an analytical gel. The gel profiles were digitized by using ImageJ software. The area of each peak was integrated and divided by the area of the corresponding peaks in the original mixture.

Protein Separation. MP-PAGE was used to purify EYFP from the crude cell extract. The preparative separation of EYFP was performed on a gel consisting of a 3 cm 12% native resolving PAG and 2 cm 50% glycerol layer with tris-glycine buffer. 300 μL of crude extract, containing ∼1.6 mg of EYFP (based on $A_{514\ nm}$)[64] was loaded onto the MP-PAGE gel. The sample was run at 150 V until the fluorescent band migrated into the glycerol layer.

The purity of the protein samples extracted either via MP-PAGE or chromatography was compared using reducing sodium dodecyl sulfate (SDS)−PAGE in tris-glycine−SDS (TGS) buffer. Fifteen microliters of each sample containing 1.0 μg of EYFP (based on $A_{514\ nm}$) in reducing sample buffer (containing dithiothreitol) was loaded onto a gel consisting of 4% stacking and 12% resolving PAGs. After 1 h of electrophoresis at 150 V, the gel was stained by CB.

The protein concentration of the extracted samples was determined from UV−visible spectra recorded using a NanoDrop 2000 instrument (Thermo Scientific). 1.3 μL portion of each sample was loaded onto the device, and the spectrum was recorded in the 250−600 nm range. Total protein concentration was determined using the BCA assay from the Pierce BCA protein assay kit (Thermo Scientific). The fluorescence spectra of the EYFP samples were measured with a Varioskan LUX Multimode Microplate Reader (Thermo Scientific).

GQD Purification. The commercial GQDs (Sigma-Aldrich, diameter <5 nm) were loaded in a three-layer gel consisting of a 20% PA separating gel. The gel was run at 150 V until the three most prominent bands were separated (2 h). TG solution (50 mM TG, pH 8.3) was used as the running buffer. The separated bands were characterized using TEM (FEI Tecnai Osiris), fluorescence spectroscopy (Varioskan), and UV−visible spectroscopy (Shimadzu 3600 Plus UV−vis−NIR spectrophotometer).

DNA−Protein/DNA−GQD Conjugate Purification. The conjugated samples were purified using a trilayer gel consisting of 12 or 15% resolving PAGs for DNA−EYFP or DNA−GQD conjugates, respectively. To assess the purity of the MP-PAGE-purified DNA−EYFP conjugate, we performed reducing SDS−PAGE for 1 h at 150 V in TGS buffer (followed by CB staining) and urea-PAGE for 45 min at 200 V in TBE buffer (followed by SG staining) for protein and DNA detection, respectively. The purity of the DNA−GQD conjugate was evaluated using native and urea denaturing PAGE for DNA.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.3c00388.

Experimental details including photographs of the experimental setup for the trilayer gel preparation and modification; original analytical PAGE profiles for purification of DNA and EYFP; chromatograms and SDS-PAGE profiles of SEC and IMAC fractions for EYFP purification; and original analytical PAGE profiles for purification of the DNA−EYFP conjugate (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Hossein Ahmadzadeh − *Chemistry Department, Faculty of Science, Ferdowsi University of Mashhad, Mashhad 9177948974, Iran;* orcid.org/0000-0002-8361-4512; Email: h.ahmadzadeh@um.ac.ir

Ardemis A. Boghossian − *Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne 1015, Switzerland;* orcid.org/0000-0001-6155-3985; Email: ardemis.boghossian@epfl.ch

Authors

Sayyed Hashem Sajjadi − *Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne 1015, Switzerland; Chemistry Department, Faculty of Science, Ferdowsi University of*

Mashhad, Mashhad 9177948974, Iran; orcid.org/0000-0003-2254-4589

Shang-Jung Wu − *Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne 1015, Switzerland;* orcid.org/0000-0003-0097-1774

Yahya Rabbani − *Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne 1015, Switzerland;* orcid.org/0000-0001-5992-9082

Vitalijs Zubkovs − *Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne 1015, Switzerland;* orcid.org/0000-0002-8769-2200

Elaheh K. Goharshadi − *Chemistry Department, Faculty of Science, Ferdowsi University of Mashhad, Mashhad 9177948974, Iran;* orcid.org/0000-0002-6789-389X

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.bioconjchem.3c00388

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Notes

The authors declare no competing financial interest.

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