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Research Letter

Analysis of Protein Binding to Liver Mitochondria by Horizontal Polyacrylamide Gel Electrophoresis Using a Converted Agarose Electrophoresis Unit

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We have previously shown that a carrier protein can be used to coat liver mitochondria for targeted transplantation to hepatocytes containing damaged mitochondria.[1] To assess binding, we initially used agarose gel electrophoresis.[2] However, this system failed to show non-bound carrier protein as a distinct band. We wondered whether polyacrylamide gel electrophoresis (PAGE) could provide better resolution. PAGE usually involves denaturation of proteins by boiling samples in sodium dodecyl sulfate.[3] All peptides become negatively charged and are separated on the basis of mass.[2] However, in our case, it was necessary to study migration of native proteins without denaturation.[4]

The use of polyacrylamide presents technical challenges not encountered with agarose. Acrylamide polymerization is distorted by contact with air, leading to surface irregularities or distortions that make migration unpredictable.[4,5] Also, because electrophoresis of native proteins may result in migration toward either the positive or negative electrode depending on the isoelectric point of the protein, the wells must be in the middle of the gel rather than at the top.[6] The aim of this research was to devise a simple method to adapt preexisting agarose gel systems to run native, non-denaturing horizontal polyacrylamide gel electrophoresis (HPAGE).

The basic commercial unit selected as a prototype for conversion to an HPAGE device was the Bio-Rad Mini-Sub Cell GT Cell (7 × 10 cm tray) agarose gel electrophoresis system containing a 15 × 10 cm UV-transparent tray, 8-well comb, casting gates, and gel caster. The key feature of the adaptive system is a hand-made plexiglass plate which, when placed on the gel tray, creates an air-free compartment when filled with acrylamide (Fig. 1). A plexiglass plate (Duco) originally 100 × 150 mm × 3.0 mm thick is cut to a 10.3 × 6.2 cm rectangle to fit into the Bio-Rad Mini-Sub Cell GT gel tray. Using a 1.0 mm diameter drill bit, a straight slot 1.0 mm wide × 56 mm long is cut across the width of the plate, 45 mm from a top edge and ending 3 mm from each end. The slot is produced to accommodate Bio-Rad GT Cell combs, which are held fast by notches on the top of the gel tray (Fig. 1A). At each corner of the plate, about 1 cm from each edge, a 3 mm diameter hole is drilled. A nylon hex nut (Avrest) with a 3 mm hole is twisted about halfway up a flat-head nylon screw (Avrest) 3 mm in diameter by 2 cm in length. A screw and nut combination is screwed into each corner hole and tightened until the desired length of the screw tip protrudes from the bottom of the plate, usually 4–6 mm. Then, the nylon hex nuts are tightened firmly against the plate to lock the screws in position (Fig. 1B). The length of exposed screws determines the thickness of the gel when poured.

The setup to prepare an HPAGE is shown in Figure 1C. The casting tray is placed in the caster on a perfectly level surface (Fig. 1D). The thickness of the gel is most easily adjusted to 3.00 mm by turning the screws and measuring the length of the protruding portion using a caliper (INSIZE 4-Way Digital, Mfr. Model 1108-150). The recommended range of gel thickness is 0.2 to 10 mm. The cover plate is placed in the casting tray, and freshly activated polyacrylamide is carefully pipetted into the slot in the cover plate. Standard precautions for handling and disposal of hazardous chemicals should be taken with the use of acrylamide. A major advantage of the horizontal PAGE setup is that once the tray is placed in the casting tray and firmly installed, the cover plate is simply placed in the tray without the need for gaskets or other methods to prevent leakage around the plate. The gel solution is added until the level reaches and completely submerges the lower surface of the cover plate. The caster is gently tapped and tilted to allow any bubbles to escape around the edges of the plate. A comb with the desired number of teeth is placed into the slot with the top fitting the existing notches provided at the top of the casting tray (Fig. 1E). After polymerization is complete, the tray with the comb in place is placed in a Bio-Rad Mini gel horizontal electrophoresis unit (Fig. 1F). The comb is removed, and samples are pipetted into the wells. The tray is transferred from the gel caster to the electrophoresis chamber, and running buffer is added to the buffer chamber. Electrical current is applied at the desired voltage (see Supplementary Table 1).

Several commercially available horizontal electrophoresis systems are intended for running agarose gels without

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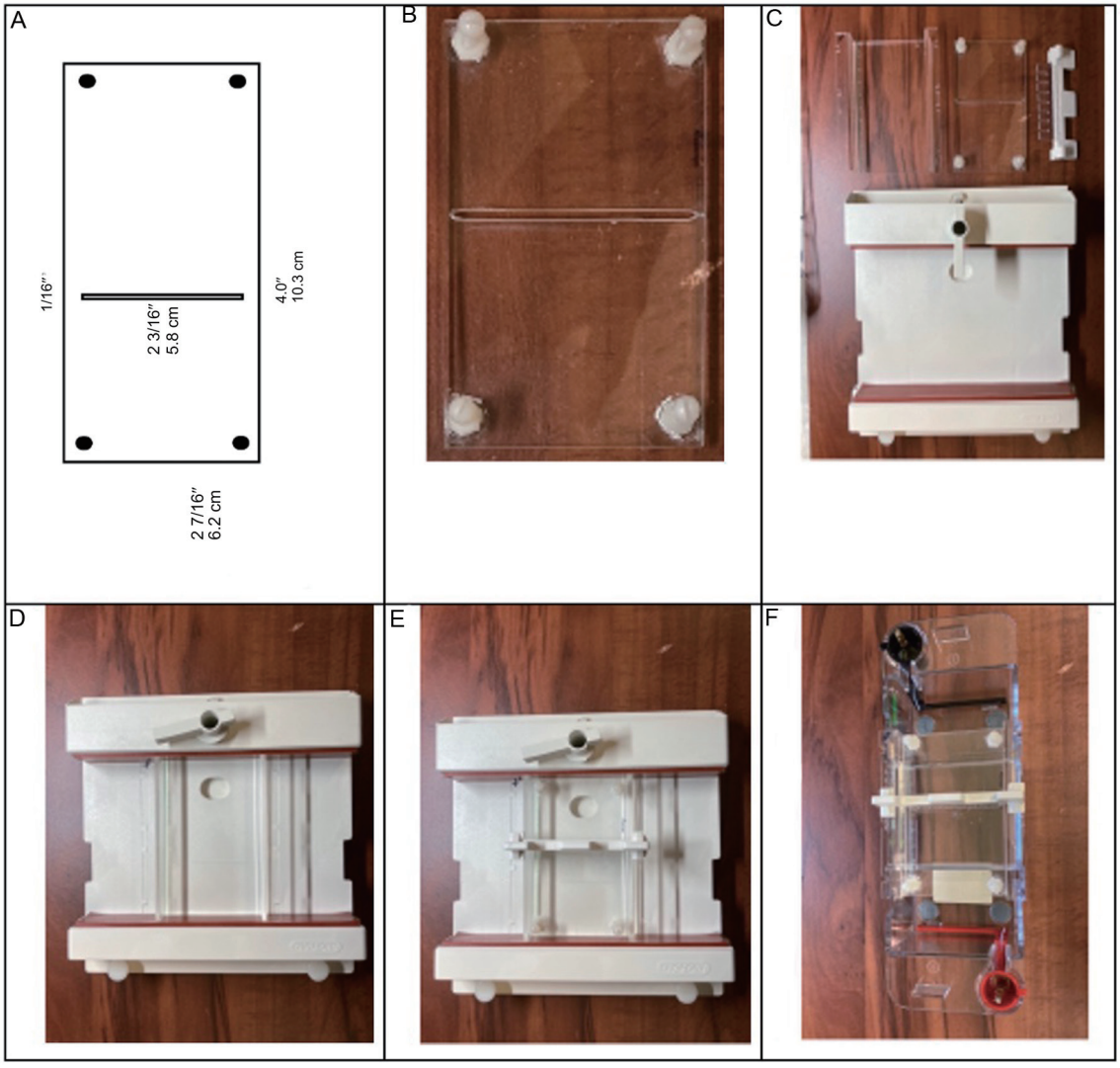

Fig. 1. Photographs of the components designed to adapt a Bio-Rad Mini-Sub Cell GT gel to perform HPAGE. (A) A diagram showing dimensions of a custom-made plexiglass cover plate with a slot cut to accommodate a comb, and four nylon screws inserted into the corners to support the plate in the polymerization tray; (B) Custom-made plexiglass cover plate; (C, top left) Bio-Rad polymerization tray (15 × 10 cm UV transparent) which holds the polymerization reaction mixture; (C, top middle) Custom-made slotted cover plate; (C, top right) Bio-Rad 8-well comb that fits into the slot of the cover plate; (C, bottom) Bio-Rad gel caster with adjustable gate; (D) Casting tray placed vertically in the gel caster and gate tightened; (E) Gel caster with tray, cover plate, and comb in place; (F) Tray, cover plate, and comb assembled in the Bio-Rad Mini gel horizontal electrophoresis chamber unit as they would appear after removal from the gel caster following acrylamide polymerization.

a cover plate. The Bio-Rad Laboratories system used in the current demonstration consists of a basic horizontal electrophoresis system including a caster, casting tray, combs, and electrophoresis unit with a list price of $562. The Cleaver Scientific system has teeth built into the caster to produce wells in the gel, but there is no way to change the number or width of the wells. The list price of the basic horizontal electrophoresis system is approximately $1,280. The Thermo Fisher Owl A-series Horizontal Gel System offers a variety of combs of varying width that hang down from the edges of the caster to produce wells in the gel. The list price of the basic Owl™ EasyCast™ B1 Mini Gel Electrophoresis Systems is $741. The BT Lab Systems offers a plastic gel caster with combs of varying sizes available to vary the number and width of wells in gels formed in a tray. The list price of the basic horizontal electrophoresis system is $620. These systems, or other horizontal gel electrophoresis systems that lack cover plates, could theoretically be converted to HPAGE as described here for an additional $20 and 2 h of labor to create cover plates.

As an example of the use of native HPAGE to analyze protein-organelle interactions, mitochondria were prepared from liver as described previously.[7] A carrier protein for targeted uptake by hepatocytes, asialoorosomucoid (AsOR), was covalently linked to polylysine (PL) as described previously,[1] except that AsOR was first labeled with Dylight 488 (Thermofisher) as described by the manufacturer. Mitochondria on ice were concentrated in PBS, pH 7.4, by centrifugation at 4 °C. Quantitation of mitochondrial mass was done by BCA protein assay (Pierce, Rockford, IL, USA).[7] To equal aliquots of mitochondria, increasing amounts of Dyl-AsOR-PL were added, making equal final volumes by addition of PBS. The complexes were incubated on ice for 45 min, after which mitochondria were separated by centrifugation at 4 °C. The supernatants were removed, and mitochondrial pellets were

Fig. 2. A photograph of a 4% HPAGE showing titration of binding of a Dylight 488-labeled asialoorosomucoid-polylysine conjugate (Dyl-AsOR-PL) to purified human mitochondria. Lanes 1–5 contained purified mitochondria, 20 μg each. Dyl-AsOR-PL in Lane 1, 0.5 μg; Lane 2, 1.0 μg; Lane 3, 1.5 μg; Lane 4, 2.0 μg; Lane 5, blank; Lane 6, 2.0 μg; Lane 7, 0.5 μg. (+) and (–) indicate the polarity of the applied electrodes during electrophoresis.

resuspended in PBS, pH 7.4, and pipetted into wells.

A 4% PAGE was prepared using the reagents listed in Supplementary Table 1. After 30–45 V for 3 h in a cold room at 4 °C, the gel was imaged using a 488 nm filter (Fig. 2), in which the negative electrode is at the top. Lanes 6 and 7 show that Dyl-AsOR-PL alone migrated upwards, leaving little in the wells. Lane 5 shows that mitochondria alone had no 488 nm fluorescence. The molecular mass of AsOR-Dyl-PL is approximately 56 kD, so it can migrate into the gel, while the mass of a mitochondrion is 5,000 to 10,000 kD, which is too large to penetrate the gel. They remain trapped in the wells (data not shown). Lanes 1–4 show that when 0.5 mg Dyl-AsOR-PL was mixed with 20 mg mitochondria, a strong fluorescent signal appeared in the wells. With increasing amounts of Dyl-AsOR-PL, the amount of fluorescence in the wells increased, indicating binding of the carrier protein to mitochondria. Excess carrier protein was seen in lanes 1–4 but was least in lane 1. Five independent replicates showed reproducibility and confirmed the titration point of 1 μg carrier to 40 μg mitochondria. Dyl-AsOR-PL alone (in the same amount as lane 1) shown in lane 7 did not produce a detectable signal in the well. Therefore, the signal in the well of lane 1 could not have been due to Dyl-AsOR-PL alone and is consistent with mitochondrial binding to Dyl-AsOR-PL in

the well.

In the adaptive system described above, a plexiglass cover plate excludes air contact during polymerization, resulting in a gel surface that is perfectly smooth and uniform in thickness. The dimensions of the cover plate are slightly smaller than the casting mold, providing a space for removal of bubbles by gently tilting the casting mold after pouring the gel. The cost of the plexiglass sheet, nylon screws, and nuts was about $20. The total time for construction of the plate was 2 h. The purpose of the nylon screws is to support the cover plate and provide a space in which acrylamide can polymerize.

However, any insert or non-metallic spacers could be used as long as they do not interfere with the electric current passing through the gel; spacers can decrease the functional size of gels. We selected nylon screws because they are inert and enable variation of gel thickness by simply lengthening or shortening the screws exposed below the plate. This is particularly convenient when analytical and preparative uses are alternately required, or when the optimal gel thickness for a study is unknown and must be determined empirically. The slot in the cover plate was intentionally placed slightly off-center to accommodate the Bio-Rad combs, which must fit into fixed grooves at the top edges of the casting tray.

The use of native HPAGE is useful for evaluating binding interactions under physiological, non-denaturing conditions. Because electrophoresis of native proteins may result in migration in either the up or down direction depending on the isoelectric point of the protein, the wells should be placed in the middle of the gel rather than at the top. Because of potential leakage of samples from mid-gel-placed wells, a horizontal PAGE system is preferred for native PAGE.

Our results do not imply that the plexiglass cover plate adaptation described here is the only or preferred method to convert a horizontal agarose electrophoresis system to an HPAGE system. Rather, they serve as a proof of principle that a slotted inert cover plate with a variable elevation above the bottom of a caster is a simple and inexpensive means of creating an HPAGE of variable thickness. The cover plate need not be plexiglass; any chemically inert rigid material could serve the same purpose. For example, glass plates have been shown to produce fine polyacrylamide gels in a horizontal orientation.[8] Glass is an excellent material for a cover plate; however, cutting a slot in thin glass to accommodate combs requires special expertise and tools. The ability to customize gel thickness by simple screw adjustment allows great flexibility, especially when both analytical and preparative (purification) uses are required at different times.

In terms of general utility of the binding study results, the use of a titration ratio determined by HPAGE fluorescence binding assay can allow maximization of mitochondrial coating and minimization of wasted excess carrier protein. This system may also be useful for studies of protein-protein, protein-nucleic acid, protein-organelle, as well as protein-cell binding properties where proteins need to be in their native state.

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Conflict of interest

GYW has been an Editor-in-Chief of *Journal of Clinical and Translational Hepatology* since 2013. He has had no role in the review process of this manuscript and has no conflicts of interest related to this publication. The other author has no conflict of interests related to this publication.

Author contributions

Proposed the concept for the review and wrote the experimental sections of the manuscript (GYW), prepared parts of the introduction, discussion, and supporting references (BT). All authors have approved the final version and publication of the manuscript.

Ethical statement

This study did not involve human participants or animal subjects; therefore, institutional review board approval and informed consent were not required.

Data sharing statement

All data generated or analyzed during this study are included in the published article or are available from the corresponding author upon reasonable request.

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