Research Article

Theme: Aggregation and Interactions of Therapeutic Proteins

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Silica Colloidal Crystals as Emerging Materials for High-Throughput Protein Electrophoresis

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Abstract. Silica colloidal crystals are a new type of media for protein electrophoresis, and they are assessed for their promise in rapidly measuring aggregation of monoclonal antibodies. The nature of silica colloidal crystals is described in the context of the need for a high-throughput separation tool for optimizing the formulations of protein drugs for minimal aggregation. The fundamental relations between molecular weight and mobility in electrophoresis are used to make a theoretical comparison of selectivity between gels and colloidal crystals. The results show that the selectivity is similar for these media, but slightly higher, 10%, for gels, and the velocity is inherently lower than that for gels due to the smaller free volume fraction. These factors are more than compensated for by lower broadening in colloidal crystals. These new media give plate heights of only 0.15 μm for the antibody monomer and 0.42 μm for the antibody dimer. The monoclonal antibody is separated from its dimer in 72 s over a distance of only 6.5 mm. This is five times faster than size-exclusion chromatography, with more than tenfold miniaturization, and amenable to parallel separations, all of which are promising for the design of high-throughput devices for optimizing protein drug formulations.

KEY WORDS: colloidal crystal; electrophoresis; high throughput; miniaturization; monoclonal antibody; plate height.

INTRODUCTION

The growth of biotechnology in the pharmaceutical industry has spurred new interest among analytical chemists for faster characterization of protein heterogeneity. Of the various undesired forms that an engineered protein can adopt, aggregation is of particular concern because aggregation occurs spontaneously in the formulated drug, causing immunogenic response in patients (1). Minimizing formation of aggregates is an essential step in formulation of protein drugs, which presently requires extensive trial and error to identify a matrix that minimizes aggregation over the time period of storage. Current techniques for characterizing protein aggregation in trial formulations have low throughput, thus creating a demand for new technology that offers higher throughput.

Separations are presently the gold standard for characterizing aggregation because the phenomenon of aggregation necessarily results in a mixture of proteins, and a physical separation facilitates quantitation of each component. Size-exclusion chromatography is widely used for such separations, but it is lower in throughput than electrophoresis because the latter allows multiple samples to be run in

parallel. The material that has dominated electrophoresis over the past half century is polyacrylamide gel. The reason why this technique is still low in throughput is that the separations themselves are slow due to the slow velocities of the protein zones and the fact that the length needs to be large to make up for zone broadening.

We are taking a new approach toward speeding up protein separations, which is to decrease the separation length drastically by reducing the broadening processes in the medium. The separations would then be faster because the length is shorter. The idea is simple: if the medium imparts little broadening to the zones, then the physical separation arising from the velocity differences would be apparent much earlier. The idea is illustrated in Fig. 1, which uses plate height as the figure of merit for broadening by the separation medium. The plate height is the amount of zone variance, σ^2 , per separation distance, L .

$$H = \frac{\sigma^2}{L} \quad (1)$$

The graphs in Fig. 1 illustrate the essential concept behind this work: two zones separate over a tenfold shorter length when the plate height is tenfold smaller. The design of a material with a smaller plate height would thus give two advantages for throughput, one being a tenfold shorter separation time,

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Fig. 1. Simulated plots of Gaussian functions vs. separation length to illustrate effect of plate height, H , on resolution. **a** For a plate height of 10 μm , the two Gaussians are unresolved in 1 cm of separation length, but they are baseline resolved in 10 cm. **b** For a plate height of 1 μm , the Gaussians are baseline resolved in only 1 cm

assuming that the velocity is not changed by the material, and the other being a tenfold more compact separation device, which would facilitate the use of microplate robotics for sample dispensing.

There are multiple factors that give rise to zone broadening during electrophoresis. The main cause is the heterogeneity of the medium. In an electrophoresis gel, the extent of cross-linking is not perfectly uniform on the microscopic scale, and the resultant velocity distribution creates a broader zone. Another factor is the distribution of velocities resulting from variations in viscosity due to Joule heating from the ionic current through the medium. Joule heating limits the voltage that can be applied, which slows the separation. Diffusion of proteins during staining and imaging also contribute to broadening and add time to the analysis.

In genomic DNA sequencing, capillary gel electrophoresis significantly increased throughput compared to slab gels (2). Soluble gels are used with this technique, where pores are created by the use of a concentrated uncharged polymer solution. Effective pore size is controlled by polymer concentration. Gel-filled capillaries inherently avoid the problem of gel heterogeneity because the polymer is in a homogeneous solution. An additional advantage is the ability to electromigrate analytes past a UV detector for quantitative detection and automation. Furthermore, higher electric fields increase speed because capillaries can be made tenfold thinner than gels, *e.g.*, 50 μm i.d. vs. 0.5 mm in thickness, which dramatically reduces zone broadening compared to slab gels (3). These factors, combined with the ability to run many capillaries in parallel, enabled capillaries to replace slab gels in the first sequencing of the human genome (2). This type of advance that increased throughput in DNA sequencing needs to be made in protein sizing.

One might think the problem for rapidly sizing proteins would be solved by adopting capillary gel electrophoresis, yet capillaries are not widely used for protein separations. The reason is that another factor besides plate height affects resolution, which is indicated in the second term of Eq. 2.

$$R_s = \frac{\sqrt{L/H}}{4} \cdot \frac{\Delta v}{v} \quad (2)$$

Resolution, R_s , is proportional to the selectivity, $\Delta v/v$, where v is the zone velocity. This equation applies to any type of separation, showing that both zone sharpness, described by the first term, and selectivity combine to give resolution, and the two effects are multiplicative. The limitation of capillary gel electrophoresis is that the selectivity is much lower because the pores are large. The lower selectivity has to be compensated by longer lengths to give the same resolution. The net effect is an increase by only about a factor of two in overall speed for capillary gel electrophoresis of proteins (4–6). Selectivity needs to be combined with a thin, homogeneous medium for faster resolution.

Nanotechnology has advanced over the same time period as biotechnology; hence, it is an auspicious time to revisit the design and development of porous media for protein separations. To circumvent heterogeneity of the porous medium for electrophoresis, we use silica colloidal crystals (7). These materials are comprised of uniformly sized silica particles (8), which can be anywhere between a few nanometers to 1,000 nm in diameter. The silica spheres are arranged to form crystals with a face-center cubic arrangement (9–11). Silica colloidal crystals have been demonstrated as media for DNA (12,13) and protein electrophoresis (12,14).

Figure 2a shows an SEM image of a silica colloidal crystal formed inside of a microchannel by 470-nm silica particles. The image shows the expected crystal faces of the face-centered cubic lattice. Such high-quality colloidal crystals have been made over wide areas (13,15). The order represented in Fig. 2a extends the 2-cm length of the channel

Fig. 2. Formats of colloidal crystals used for electrophoresis. **a** SEM image of channel cross section showing high crystallinity. Image from Birdsall and Wirth, permission requested, © 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. **b** SEM image of the top of the colloidal crystal in the channel, showing ordered arrangement of silica spheres. The *expanded view* shows the pores formed among the spheres. **c** Photograph of a set of parallel channels, which are 2 cm in length. **d** Photograph of the end of a capillary packed with colloidal crystals, with SEM image superimposed to show polycrystalline packing, taken from Wei *et al.* (16) with permission, Copyright © 2012, American Chemical Society

in the device shown in Fig. 2c. Figure 2b shows an SEM image of the top surface of the colloidal crystal, which has the characteristic hexagonal geometry of the [111] crystal face. Pores for sieving exist everywhere where particles meet, and one can see from the expanded view in the inset of Fig. 2b that these pores are tens of nanometers, which are suitable for sieving or the size exclusion of proteins. Colloidal crystal can also be formed in capillaries (17–20), and Fig. 2d shows a photograph of a capillary packed with a silica colloidal crystal, along with an SEM image of the same capillary. The opalescence owes to the lattice spacing being on the order of the wavelength of light. Gem opals are also comprised of colloidal silica in face-centered cubic lattices, giving similar SEM images (21). The color is shifted when the index of refraction of the medium changes, and this so-called photonic effect is a means of detecting biomolecules without labeling (22), which might be useful in optimizing formulations.

The purpose of this work is to model an electrophoretic separation of a monoclonal antibody and its dimer using data and equations from the literature, and to carry out such a separation using a silica colloidal crystal for which the particle diameter is selected based on the modeling. Sodium dodecyl sulfate (SDS) denaturing is used to impart a charge on the monoclonal antibodies, which is necessary because these proteins typically have *pI* values near neutral pH. Covalent aggregation is ubiquitous for monoclonal antibodies due to scrambling of disulfide bonds. The technology

would be applicable to characterizing covalent aggregates since SDS breaks up noncovalent aggregates. The plate height will be measured to assess speed and miniaturization.

METHODS

A sample of pharmaceutical-grade monoclonal antibody, obtained from Eli Lilly, had gone through drug development but is no longer an active candidate. The antibody was labeled with AlexaFluor 546 (Invitrogen, Carlsbad, CA) according to the vendor's instructions. The antibody had been given a 10-min heat treatment at 75°C to promote aggregate formation, and size-exclusion chromatography confirmed that a small amount of dimer was formed. A concentration of 0.5 mg/mL was prepared in SDS and electrophoresis buffer, as described earlier (12,14).

Uniform nonporous silica particles were obtained from Fiber Optic Center (New Bedford, MA); these were calcined and annealed as described previously (14). The silica surfaces were chemically modified to bear linear polyacrylamide chains, which were grown from the surface by using surface-initiated atom-transfer radical transfer polymerization (23). The material thus mimics a polyacrylamide gel, but is thinner and more homogeneous. The details of how to prepare the silica colloidal crystal in capillaries have been published (16), as well as the details of how to perform electrophoresis (14).

Fig. 3. Electrophoresis data for four proteins using a silica colloidal crystal as the medium. Proteins A–D are lysozyme, trypsin inhibitor, carbonic anhydrase, and bovine serum albumin, respectively. **a** Electropherogram of the proteins, with data points (circles), and a fit to four Gaussians (line). **b** Plot of electrophoresis data using Eq. 3. Pore radius is indicated by ξ . Both panels are adapted from Birdsall *et al.* (14) with permission requested, © 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

RESULTS AND DISCUSSION

Electrophoresis results reported recently by our group (14) are analyzed further here to understand how these materials can be used to study aggregation of monoclonal antibodies. Figure 3a shows an electropherogram, where a silica colloidal crystal made of a particle 483 nm in diameter was used for electrophoresis of lysozyme (14.7 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), and bovine serum albumin (67 kDa). The electropherogram shows narrow zones as the proteins electromigrate past a fixed detection point, which was only 4 mm from the injection point. The zones broaden as protein size increases. This is typical behavior for electrophoresis, where plate height increases with protein radius. The plate heights were not calculated in the original work, but are essential in the context of the present work, and thus are calculated here: the plate heights increase in the order 0.33, 1.0, 1.6, and 3.3 μm for the smallest to the largest protein. These are all smaller than the typical plate height of 10 μm for medium-sized proteins in gels (24). The unusually small plate heights demonstrate that the silica colloidal crystals are promising for miniaturization. Figure 3b shows a plot of molecular weight vs. normalized migration rate through the medium. The shape of the curve is similar to such curves for gel electrophoresis, where the mobility goes to zero when the molecular weight exceeds the pore size, and the velocity is no longer dependent on molecular weight when the protein radius becomes negligible in size compared to the pore radius. The solid curve is a plot of the predicted migration rate using Eq. 3, which was derived from first principles (14), and each parameter was independently characterized.

$$\frac{\mu}{\mu_0} = \varepsilon \left(\frac{\xi - R}{\xi} \right)^2 \quad (3)$$

The mobility, μ , in the medium is normalized to the mobility in free solution, μ_0 , which was measured by open capillary electrophoresis. The pore radius, ξ , and porosity, ε , were independently determined from flow rate measurements. The protein radius is presented by R . Since velocity is proportional to mobility, then $\Delta\mu/\langle\mu\rangle$, is equivalent to the ratio $\Delta v/\langle v\rangle$ used in

Eq. 2 for expressing selectivity. The important outcome is that we can use Eq. 3 to predict the selectivity, $\Delta\mu/\langle\mu\rangle$, for other proteins, *i.e.*, monoclonal antibodies and aggregates, and other pore radii that are more optimal for the larger proteins.

For comparison of selectivity with gels, the theoretical treatment by Kopecka *et al.* (25) provides an equation to describe gel electrophoresis.

$$\frac{\mu}{\mu_0} = \exp\left(-\frac{\pi}{4} \left(\frac{R+r}{\xi}\right)^2\right) \quad (4)$$

Fig. 4. Gel electropherogram of a monoclonal antibody initially (lane 4) and after storage (lanes 5–9). The size standards are shown in lane 2. The figure is adapted from Mahler *et al.* (26) with permission requested, © 2013 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Fig. 5. Comparison of selectivity for gels and colloidal crystals in protein electrophoresis. **a** Data points (red circles) for the size standards of the gel in Fig. 4 and fit to Eq. 4 (red line). Plot of Eq. 3 (blue line, blue circles). **b** Theoretical plots for gel (red line) and colloidal crystal (blue line) for pore sizes optimized for resolving an antibody from its dimer. In both panels, the antibody molecular weight (MW) (150 kDa) and its dimer MW (300 kDa) are indicated by the dotted lines

In this equation, r is the radius of the gel fibers, *e.g.*, the polyacrylamide chains, and the other terms have the same meanings as for Eq. 3. To demonstrate that Eq. 4 is valid, data from the literature are used. Figure 4 shows a separation of a monoclonal antibody and its aggregates and fragments by gel electrophoresis, published by Mahler *et al.* (26). The image illustrates the benefit of gel electrophoresis: a wide range of molecular weights is separated, and many lanes can be run simultaneously. The first lane, which has the size standards, allows one to identify the antibody, which is the most intense

band in the subsequent lanes. In the subsequent lanes, the aggregates of the antibody are visible at higher molecular weight, and fragments are visible at lower molecular weights. The data for the size standards are analyzed here by Eq. 4, and the results are plotted in Fig. 5a. The data in red are for the gel, and the curve is from Eq. 4, with r and ξ adjusted for best fit.

Figure 5a also uses Eq. 3 for colloidal crystals to make a comparison of selectivity, which is the blue curve. The circles show the theoretical positions of the same size standards for

Fig. 6. **a** Plot of both selectivity (solid line) and relative mobility (dotted line) as a function of pore radius to show the trade-off. **b** Plot of plate height vs. the square of the broadening function of Eq. 5 for a pore radius of 27 nm. The line is least-square fits of data points

comparison. The mobility was divided by porosity to make the x -axes coincide. The graph reveals that the selectivity is similar for the gel and the colloidal crystal, with selectivity being somewhat higher for the gel.

The two equations allow a theoretical comparison between a gel and a colloidal crystal when each have a pore size better suited for resolving a monoclonal antibody from its dimer with higher velocity. The resulting plot is in Fig. 5b, where the pore sizes were adjusted to give the same velocity for the dimer. The comparison shows that the gel has a 10% higher selectivity. This is a small difference, but it seems surprising that the disordered gel would give higher selectivity. The underlying reason is that the pore size distribution enables the gel to separate a wider range of proteins, which translates to a smaller slope in $\log(MW)$ vs. mobility. The analysis shows that the colloidal crystal is a reasonable substitution for the gel from the standpoint of selectivity.

Since the goal is high throughput, the velocities must also be considered. In Fig. 5, the plots were made with respect to

“relative mobility,” which is the normalized mobility divided by the porosity. The porosity is not known for the gel, but it is likely close to unity since the polyacrylamide fibers probably take up much less of a negligible volume fraction of the gel. In fact, the best fit of the data for the size standards gave a fiber radius of zero. For the colloidal crystal, the porosity is 0.2, and the porosity is inherently low because the silica particles take up most of the volume. What this means in practice is that the absolute velocity in the colloidal crystal will be about fivefold lower than that of a gel. The colloidal crystal thus has two problems to overcome: somewhat smaller selectivity and a much lower velocity. These limitations can potentially be overcome by a much smaller plate height.

In choosing the particle diameter for the colloidal crystal, one must consider the trade-off between speed and selectivity. To elaborate, Eq. 3 shows that selectivity is enhanced by having the pore size closer to the protein size, but the equation also shows that the electrophoretic mobility is slowed as the pore size approaches the protein size. Equation 3 can be used to examine

the trade-off between speed and selectivity to select an optimal pore size. A plot of both selectivity and speed with respect to pore size was made by use of Eq. 3 and is given in Fig. 6a. The plot reveals that selectivity drops sublinearly whereas speed goes up nearly linearly until the pore radius exceeds 30 nm. These considerations would suggest a pore radius of 30 nm to be a good compromise.

Zone broadening is also a consideration in selecting pore size since the plate height increases strongly with protein size for a given pore size. Such behavior indicates that there is a pore size distribution. One can model the dependence of plate height on protein radius by introducing a stochastic variable, ξ' , to account for random changes in pore radius, and relating this to changes in μ . Adding ξ' to ξ in Eq. 3 and then differentiating mobility with respect to ξ' gives a simple relation when $\xi \gg R \gg \xi'$.

$$\frac{\partial(\mu/\mu_0)}{\partial \xi'} = \epsilon \left(\frac{2R}{\xi^2} \right) \quad (5)$$

Since this variation in mobility with pore size would be proportional to zone width, then its square would be proportional to plate height. If the pore size distribution is the origin of the broadening, and the pore size variations are small, then plate height should be proportional to R^2 . Such a plot is given in Fig. 6b, where the least squares fit shows good agreement, and the intercept is zero. Extrapolating to higher molecular weights gives calculated plate heights of 9, 20, and 32 μm for monomer, dimer, and trimer of a monoclonal antibody. For detecting aggregation of antibodies, larger pores are needed.

We chose 1,000 nm as the particle diameter, which is twice that of the 500-nm particles that gave the pore radius of 27 nm. One cannot assume the new pore radius is also twice as large because the polymer chains grow longer in larger pores. We perform electrophoresis to determine whether small plate heights are now observable for monoclonal antibodies and aggregates. Figure 7a shows an image from the separation of a monoclonal antibody and its dimer. The brighter monomer peak is separated from the dimer peak over a length of only 6.5 mm and in a time of only 72 s. Based on Gaussian fits of the two zones, the calculated plate heights are 0.15 and 0.42 μm for the monomer and dimer, respectively. These extremely low plate heights are extraordinary for monoclonal antibodies in any type of separation. Wider pore gels have been used to separate monomers from dimers of monoclonal antibodies, but the broadening is severe (27). Figure 7b shows a plot of the packed capillary electrophoresis data of Fig. 7a, where a fixed detection position of 0.65 cm was used and the intensity was monitored vs. time to generate an electropherogram. For comparison, Fig. 7c shows a chromatogram from size-exclusion chromatography for the same sample. The resolution is similar, slightly higher for electrophoresis, but the time scale for electrophoresis is fivefold shorter. Both separations show primarily the monomer and dimer peaks. There is evidence for a large aggregate in the electrophoresis data. Both show evidence of species with smaller molecular weight, but more precisely controlled samples will need to be studied as well as a variety of particle sizes, to learn what additional information is obtainable. The

Fig. 7. Separation of monoclonal antibody monomer and dimer. **a** False-color fluorescence image of antibody followed by its dimer as they electromigrate in SDS and buffer through a 100- μm -i.d. capillary packed with silica colloidal crystal and reach a separation length of 6.5 mm in 72 s. **b** Time trace of the same separation as in panel **a**. **c** Size-exclusion chromatogram of the same protein sample as in panels **a** and **b**

fivefold higher speed in separating monomer from dimer, combined with the present commercial capability of running 24 capillaries simultaneously in electrophoresis, promises higher throughput for assessing aggregation of monoclonal antibodies in the optimization of formulations.

CONCLUSIONS

Silica colloidal crystals are an emerging material for fast separations of monoclonal antibodies and aggregates. The uniformity of the material allows a much smaller footprint and a higher speed. With the tiny separation lengths of less than 1 cm, the media could be patterned for direct compatibility with the robotics of 96-well plates for high throughput. The results presented here use SDS-denatured protein, which assesses covalent aggregates, which are arguably more deleterious than noncovalent aggregates. Further studies are needed to address native electrophoresis for assessing noncovalent aggregates. The silica colloidal crystal makes it easy to control pore size for sieving large proteins with minimal broadening. Larger particles could be used, in principle, to assess even larger aggregates. The remaining issues for reduction to practice are the design of such devices and label-free detection.

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REFERENCES

1. An Z. Therapeutic monoclonal antibodies from bench to clinic. Hoboken: Wiley; 2009.
2. Dovichi NJ, Zhang JZ. How capillary electrophoresis sequenced the human genome. *Angew Chem-Int Edit*. 2000;39(24):4463–8.
3. Jorgenson JW, Lukacs KD. Zone electrophoresis in open-tubular glass-capillaries. *Anal Chem*. 1981;53(8):1298–302.
4. Gomis DBL, Junco S, Exposito Y, Gutierrez D. Size-based separations of proteins by capillary electrophoresis using linear polyacrylamide as a sieving medium: model studies and analysis of cider proteins. *Electrophoresis*. 2003;24(9):1391–6.
5. Guttman A, Nolan J. Comparison of the separation of proteins by sodium dodecyl-sulfate slab gel-electrophoresis and capillary sodium dodecyl-sulfate gel-electrophoresis. *Anal Biochem*. 1994;221(2):285–9.
6. Zhu ZF, Lu JJ, Liu SR. Protein separation by capillary gel electrophoresis: a review. *Anal Chim Acta*. 2012;709:21–31.
7. Colvin VL. From opals to optics: colloidal photonic crystals. *MRS Bull*. 2001;26(8):637–41.
8. Stober W, Fink A, Bohn E. Controlled growth of monodisperse silica spheres in micron size range. *J Colloid Interface Sci*. 1968;26(1):62–9.
9. Jiang P, Bertone JF, Hwang KS, Colvin VL. Single-crystal colloidal multilayers of controlled thickness. *Chem Mat*. 1999;11(8):2132–40.
10. Miguez H, Meseguer F, Lopez C, Mifsud A, Moya JS, Vazquez L. Evidence of FCC crystallization of SiO₂ nanospheres. *Langmuir*. 1997;13(23):6009–11.
11. Pusey PN, Vanmegen W, Bartlett P, Ackerson BJ, Rarity JG, Underwood SM. Structure of crystals of hard colloidal spheres. *Phys Rev Lett*. 1989;63(25):2753–6.
12. Zeng Y, Harrison DJ. Self-assembled colloidal arrays as three-dimensional nanofluidic sieves for separation of biomolecules on microchips. *Anal Chem*. 2007;79(6):2289–95.
13. Zeng Y, He M, Harrison DJ. Microfluidic self-patterning of large-scale crystalline nanoarrays for high-throughput continuous DNA fractionation. *Angew Chem-Int Edit*. 2008;47(34):6388–91.
14. Birdsall RE, Koshel BM, Hua Y, Ratnayaka SN, Wirth MJ. Modeling of protein electrophoresis in silica colloidal crystals having brush layers of polyacrylamide. *Electrophoresis*. 2013. doi:10.1002/elps.201200413.
15. Jiang P, Prasad T, McFarland MJ, Colvin VL. Two-dimensional nonclose-packed colloidal crystals formed by spincoating. *Appl Phys Lett*. 2006;89(1).
16. Wei BC, Rogers BJ, Wirth MJ. Slip flow in colloidal crystals for ultraefficient chromatography. *J Am Chem Soc*. 2012;134(26):10780–2.
17. Kamp U, Kitaev V, von Freymann G, Ozin GA, Mabury SA. Colloidal crystal capillary columns—towards optical chromatography. *Adv Mater*. 2005;17(4):438–43.
18. Malkin DS, Wei BC, Fogiel AJ, Staats SL, Wirth MJ. Submicrometer plate heights for capillaries packed with silica colloidal crystals. *Anal Chem*. 2010;82(6):2175–7.
19. Moon JH, Kim S, Yi GR, Lee YH, Yang SM. Fabrication of ordered macroporous cylinders by colloidal templating in microcapillaries. *Langmuir*. 2004;20(5):2033–5.
20. Wei B, Malkin DS, Wirth MJ. Plate heights below 50 nm for protein electrochromatography using silica colloidal crystals. *Anal Chem*. 2010;82(24):10216–21.
21. Egas DA, Wirth MJ. Fundamentals of protein separations: 50 years of nanotechnology, and growing. *Annu Rev Anal Chem*. 2008;1:833–55.
22. Pal S, Fauchet PM, Miller BL. 1-D and 2-D photonic crystals as optical methods for amplifying biomolecular recognition. *Anal Chem*. 2012;84(21):8900–8.
23. Huang X, Wirth M. Surface-initiated radical polymerization on porous silica. *Anal Chem*. 1997;69:4577–80.
24. Papautsky I, Mohanty S, Weiss R, Frazier AB. High lane density slab-gel electrophoresis using micromachined instrumentation. *Electrophoresis*. 2001;22(18):3908–15.
25. Kopecka K, Drounin G, Slater G. Capillary electrophoresis sequencing of small ssDNA molecules versus Ogston regime: fitting data and interpreting parameters. *Electrophoresis*. 2004;25:2177–85.
26. Mahler HC, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. *J Pharm Sci*. 2009;98(9):2909–34.
27. Slavickova A. Electrophoresis of nonreduced IgM and IgG monoclonal-antibodies on mixed agarose-polyacrylamide gels. *Electrophoresis*. 1990;11(10):892.