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The assessmentof the effect of acrylamide concentration on the diffusion of biomolecules in a gel

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Received February 28, 2024
Revised June 7, 2024
Accepted June 23, 2024

A highly accurate method for quantifying target DNA molecules is the implementation of an amplification reaction in a thin layer of polyacrylamide gel.The gel impedes the diffusion of DNA molecules, which leads to the concentration of amplification products (amplicons) around the original target molecule and the formation of molecular colonies. Increasing the concentration of acrylamide produces denser gels, which reduces colony size. Reducing the size of colonies while maintaining the effective gel area allows you to record a larger number of colonies, increasing the dynamic range of the method. To study the effect of acrylamide concentration on the diffusion of amplicons (DNA fragments obtained as a result of the analysis) in the gel, a simple method was used to estimate the diffusion coefficient based on recording the moving boundary of fluorescently labeled biomolecules. An experimental assessment of the effect of acrylamide concentration on the diffusion of synthetic oligonucleotides and amplicons in the gel shows that when the acrylamide concentration increases from 7 to 10%, the estimated diffusion values change by approximately 2 times.

Keywords: microfluidic chip, polyacrylamide gel, diffusion coefficient, amplicon.

DOI: 10.61011/TP.2024.09.59291.56-24

Introduction

Microfluidic chips are widely used in devices for a polymerase chain reaction (PCR). One of the varieties of their use is the PCR method in a thin layer of polyacrylamide gel located in the reaction chamber of a microfluidic chip, which provides a number of advantages over the liquid version [1–4]. In a microfluidic chip, a thin layer of polyacrylamide gel is used as an integrated functional structure of the medium for conducting PCR using the molecular colony technique (MCT). When conducting a DNA amplification reaction (for example, PCR) in a thin layer of polyacrylamide gel [1], the gel structure immobilizes the long target macromolecules of the DNA sample, but does not affect the diffusion of the reaction components. At relatively low concentrations of target molecules, the latter are located at a significant (compared to the size of the molecules) distance from each other. The reaction products (amplicons) accumulate around the original DNA in the form of a colony. The count of colonies that are spatially separated in the gel allows for direct quantitative analysis.

Obviously, the maximum number of recorded colonies is limited by the area of the gel in the reaction chamber. In the case of overlapping or merging colonies, quantitative analysis becomes impossible. Reducing the size of colonies while maintaining the gel area increases the number of „accommodated“ colonies, which increases the dynamic range of the method and reduces the likelihood of colony overlap. It is known that with an increase of the concentration of acrylamide in the gel, for example, from 6 to 15%, the average radius of colonies can change from 300 to 50 μm (for amplicons with a length of 234 base pair) [2]. To use thin-layer gels as functional structures of microfluidic chips, the topology and geometric parameters of the reaction chamber should be set and the characteristics of the gel layer should be known. To this end, the present work investigates the effect of acrylamide concentration on the diffusion of amplicons (DNA fragments) in a polyacrylamide gel.

Gel is a dispersed system consisting of a liquid dispersion medium and a high-molecular dispersed phase that forms a spatial porous structure. However, it would be wrong to consider the gel as a regular spatial lattice with rigid cells of a certain average size. In the case of a small amount of crosslinking agent the gel comprises rather long strands (fibers) with a small amount of crosslinking agent that fill the entire volume and are randomly stitched together only at certain points. Such a system is not rigid. Macromolecules migrating in the gel can push apart flexible long sections of linear acrylamide polymers, which consumes energy, thus, the migration of molecules slows down and a kind of „friction“ against the gel occurs [5]. The higher the acrylamide content, greater the number of polymer filaments, the smaller the gaps between them and the stronger the friction.

The task of estimating the diffusion coefficients of various substances in gels is relevant, for example, for practical purposes of creating bioreactors based on gels [6]. Hydrogels are biocompatible, as well as structurally and in

composition similar to the extracellular matrix, therefore, tissue engineering techniques using microgel droplets are being developed to create artificial tissues [7,8]. In existing models, complex calculation algorithms are used, and inorganic substances (indicators, dyes) and components of solutions necessary for cell growth are used as research objects [9]. The effect of acrylamide concentration on the diffusion of biomolecules representing DNA fragments of different lengths is studied in this paper. It is proposed to use a simple method of estimating diffusion for this purpose.

1. Materials and methods

Polyacrylamide gels obtained by copolymerization of acrylamide (AA) and bis-acrylamide (MBA, N,N′-methylene bisacrylamide) in the presence of a polymerization initiator (ammonium persulfate) and a catalyst were used to assess the effect of acrylamide concentration on the diffusion of amplicons in the gel. Two concentrations of acrylamide — 7 and 10% (w/w) were used to obtain gels of different densities. Further in the work, these gels are designated as 7% and 10% gel, respectively. Since the structure of the gel can change over time [10], freshly prepared gels were used for experimental studies.

Since polyacrylamide gel is an optically transparent medium, the so-called moving boundary method can be used for experimental evaluation of diffusion, when the rate of advance into the polymer matrix of the front (boundary, plane) of a colored diffusant with a constant concentration is estimated [11]. At the initial stage, a dye solution (black water-based ink) was used as a portable substance for a preliminary assessment of the diffusion properties of gels of different densities. A dye solution was applied to the finished gel samples, then the samples were sealed and stored vertically at room temperature. The movement of the dye front in the vertical direction (up or down) in gels of different concentrations was recorded for 5 min after applying the dye and measurements were periodically repeated for two days. The diffusion distance was estimated using digitized photographs of samples with the boundary of the dye front in the gel.

The advantages of the technique used are simplicity, clarity, lack of need for specialized equipment or destruction of the sample, the disadvantages include low accuracy. In contrast to a similar technique used in the paper [10], when capillaries with gels based on silicic acid salt closed from the upper end were immersed with the lower part in a contrast dye solution, a fixed volume of dye was applied to the gel cylinders in our case , after which the gel-dye system was sealed. This is due to the desire to further prevent or reduce contamination of work surfaces when working with amplicon solutions. In this case, the volume of the injected analyte is finite, therefore, the observed processes may not fully correspond to the kinetics of movement of isoconcentration planes, which is assumed in the method of moving boundaries.

The gel sample is in a glass capillary sealed on both sides with polymer caps.

DNA fragments labeled with a fluorescent dye were used in experimental studies for evaluation of the effect of acrylamide concentration on amplicon diffusion. Amplicons with a length of 103 base pair (bp) were obtained by real-time PCR in the presence of the dye SYBR Green I (reagent kit M-427, Syntol LLC, Russia) and specific primers (DNA Synthesis LLC, Russia). Device ANK-32 (IAI RAS, Russia) was used for the reaction, synthesized fragments of cytokeratin-19 cDNA of known concentration were used as a DNA target. The work also used short synthesized single-stranded DNA fragments with a length of 27 nucleotides with FAM dye (Syntol LLC, Russia).

Gel samples were prepared in glass capillaries (see Figure) with an inner diameter of ~ 3 mm in two stages for estimation of the diffusion movement of fluorescent dye labelled DNA fragments: a polymerizing gel containing labeled DNA fragments was layered on the lower freshly prepared gel layer.

The movement of the front of labeled DNA fragments into the lower gel layer was recorded using a prototype of DMC-1 fluorescent detector (IAI RAS, Russia), with a laser excitation source wavelength of 473 nm. The front boundary was determined by changing the fluorescence intensity along the capillary axis, the threshold value was calculated at the level of 5% of the initial fluorescence signal. Measurements were carried out for a week at room temperature (24°C), all gels remained transparent during the entire duration of the experiment, gel drying was not observed. The total time of the experiments was: for DNA fragments with a length of 27 nucleotides — 1 day, for dye — 2 days, for fragments with a length of 103 bp — 3 days. A special long-term (up to 6 days) study of fragments with a length of 27 nucleotides in dense gels (10% gel) was also carried out.

2. Results and discussion

2.1. Movement of the dye front in gels

Preliminary experiments made it possible to record the difference in the diffusion movement of the dye front (black ink) in gels with different concentrations of acrylamide [12]. According to the Einstein-Smoluchowski formula, it is possible to indirectly estimate the diffusion coefficient D, knowing the measurement time (t) and the diffusion displacement ($\overline{\sigma}$) of the dye front

$$\delta = \sqrt{2Dt}. \quad (1)$$

Thus, the diffusion coefficient of the dye in a 7% gel can be defined as $(1.9 \pm 0.1) \cdot 10^{-9}\,\mathrm{m^2/s}$, and $(1.3 \pm 0.2) \cdot 10^{-9}\,\mathrm{m^2/s}$ in a 10% gel. The obtained values are comparable with experimental estimates of the diffusion coefficient of water-based inks $(0.96 \cdot 10^{-9}\,\mathrm{m^2/s})$ in gels made of silicic acid salt in Ref. [10]. Also, these values are in good agreement with the statement from Ref. [13] that with strong swelling of the gel, the diffusion coefficient is much higher than the usual values and is close to the diffusion coefficients of small molecules and ions in water ($\sim 10^{-9}\,\mathrm{m^2/s}$).

Since the dependence of the movement of the dye front on time (t) can be represented as: $\overline{\sigma} \approx t^a$, then the approximation of experimental data in double logarithmic coordinates for the gels used allowed estimating the parameter a: 1) 0.62 ± 0.03 in case of a downward movement of the dye; 2) 0.49 ± 0.02 in case of an upward movement. The coefficient of determination exceeds 0.88 for constructing approximations. The number of time counts was 6 (from 1.5 to 44 h), each has at least three repetitions. Since the value of the parameter $a = 1/2$ corresponds to diffusion distance (1), then it can be concluded that experimental data confirm the determining contribution of diffusion. The direction of movement of the front does not have a significant effect on this.

2.2. Movement of the front of labeled amplicons in gels

When evaluating the diffusion coefficient of amplicons, it should be borne in mind that the movement of sufficiently short DNA fragments (up to 200 nucleotides long) is studied in this paper. Their length is comparable to the so-called persistence DNA length (46–50 nm). In this case, the DNA fragment can be considered as an inflexible rectilinear rod (diameter ~ 2 nm).

The Stokes-Einstein formula in the spherical particle approximation can be used in (1) to estimate the diffusion coefficient D:

$$D = \frac{k_B T}{6\pi\eta r}, \quad (2)$$

where k_B — Boltzmann constant value, T — absolute temperature, η — the coefficient of dynamic viscosity (for polyacrylamide gel $1.21 \cdot 10^{-3}$ Pa·s), r — the radius of the particle.

Table 1. Experimentally obtained front displacement for amplicons with a length of 103 bp in gels made with different concentrations of acrylamide

Time, h	Displacement of the amplicon front boundary, mm (coefficient of variation, %)	
	7% gel ($n = 3$)	10% gel ($n = 3$)
20	1.33 ± 0.05 (4%)	0.78 ± 0.06 (8%)
38.5	–	1.24 ± 0.05 (4%)
45	1.9 ± 0.1 (6%)	1.4 ± 0.1 (8%)
68.5	2.5 ± 0.2 (7%)	1.7 ± 0.1 (6%)

The radius of inertia of the rod $r = l/\sqrt{6}$ is taken as the radius of an equivalent spherical particle, where l — the length of the rod is proportional to the number of nucleotides N of the DNA fragment (the length of one nucleotide is assumed to be 0.34 nm). The equivalent radius of r will be 14.3 nm for a DNA fragment with a length of 103 base pairs and it will be 3.7 nm, respectively, for fragments with a length of 27 nucleotides.

If we consider convective motion (precipitation under the influence of gravity) and compare it with the diffusive movement of oligonucleotides, then the comparison of their contribution is determined by a coefficient that makes sense of the characteristic Peclet number:

$$Pe = \frac{\pi d^3 L(\rho^* - \rho_0)}{6kT} g,$$

where d — the equivalent diameter of inertia calculated based on the approximation of a rectilinear rod (28.5 nm for a fragment of 103 bp), L — the characteristic length equal to the radius of the capillary, ρ_0 — the density of the medium (gel), equal to $1.2\,\mathrm{g/cm^3}$, ρ^* — the density of the oligonucleotide (based on E.coli DNA, $\rho^* = 1.69{-}1.73\,\mathrm{g/cm^3}$), g approximately $9.8\,\mathrm{m/s^2}$.

Accordingly, the Peclet number is about 0.022, which makes it possible to neglect convection and assume the dominance of the diffusion movement of oligonucleotides during the movement of the amplicon front in the gel. The true value of this indicator may differ from the calculated value by 2–3 times if the equivalent inertia diameter is not correctly and adequately estimated when calculating the Peclet number in the formula (2). However, this circumstance will not affect the validity of the hypothesis of the dominance of diffusion over convection.

The experimental values obtained in gels of different densities are given in Table 1. Let us use the formula (1) to estimate the value of the diffusion coefficient based on the value of the diffusion displacement. The relative error in calculating the diffusion coefficient after 6 days was less than 1.7% according to the estimation of the relative error of indirect measurements based on the measurement errors of distance (1 pixel = 24 μm) and time (error 1 h). At the same

time, the calculated parameter a of the power dependence corresponding to the tangent of the angle of inclination in double logarithmic coordinates for three repetitions of all measurements for 7% gel is 0.54 ± 0.03 with a coefficient of determination of 0.977, which indicates a very high degree of linearity. The parameter a is 0.52 ± 0.05 with a determination coefficient of 0.869 for a denser 10% gel. Thus, it can be concluded that the data obtained correspond to the hypothesis of diffusion displacement according to the square root law.

According to estimates of the calculated values, the diffusion coefficient for fragments with a length of 103 bp in a 7% gel is $(1.2 \pm 0.1) \cdot 10^{-11}$ m^2/s ($n = 12$), and it is defined as $(0.5 \pm 0.1) \cdot 10^{-11}$ m^2/s ($n = 15$) in a 10% gel.

Let us use the approach of determining the diffusion coefficient by the speed of movement of the boundary of the constant concentration of the authors of the paper [14] as an alternative estimation. In this case, the limit is set for the ratio δ/L (where L — half the thickness of the sample), which should lie in the range from 0.4 to 0.9 [11]. Comparing the data at two points, both of which satisfy the necessary conditions, makes it possible to exclude concentration data, thereby solving the equation to determine D

$$\ln(\cos(\pi(L-\delta_1)/2L) - \ln(\cos(\pi(L-\delta_2)/2L) = \pi^2 D(t_1 - t_2)/(4L^2). \quad (3)$$

In our case ($L = 1.5$ mm), measurements made in a denser 10% gel after two days are suitable for analysis, i.e. 0.78, 1.24 and 1.40mm, which corresponds to 0.5, 0.8 and 0.9 of half the thickness of the sample. The last measurement slightly exceeds the upper recommended limit, so it is possible to assume that there is a slight calculation error.

The estimate according to this formula for two segments from the starting point (0.78 mm) corresponds to $(0.35 \pm 0.05) \cdot 10^{-11}$ m^2/s, which is comparable with previous estimates by another method $(0.5 \pm 0.1) \cdot 10^{-11}$ m^2/s.

According to the third method of estimating diffusion in the spherical particle approximation, the calculated diffusion coefficient for this model corresponds to $1.25 \cdot 10^{-11}$ and $1.17 \cdot 10^{-11}$ m^2/s. It is possible to estimate the radius of inertia of the amplicon based on previous close estimates, using the Stokes-Einstein formula and analyze the applicability of the equivalent sphere model with respect about 100 bases long in a 10% gel. Since the dynamic viscosity of the medium corresponds to $1.3 \cdot 10^{-3}$ Pa·s, the obtained estimate of the equivalent inertia diameter turns out to be greater than the total length of the oligonucleotide and is on the order of 100 nm, i.e. approximately 3–4 fold difference of the obtained estimates. Thus, model of equivalent sphere for such a DNA fragment can only be used for very approximate estimation calculations.

An experimental estimation of the effect of acrylamide concentration on the diffusion of amplicons in the gel shows that the estimated diffusion values (according to a power function) change in 2.3 times with an increase of acrylamide from 7 to 10%. The ratio of radii of molecular colonies in relation to amplicons with a length of 120 bp was indicated for 6% and 10% gels in Ref. [2], where the size of molecular colonies (polonies) in gels of different densities was compared. The ratio of colony radii (diffusion distances) of approximately 1.65 was determined using interpolation and the calculated estimate obtained for the 7% concentration of acrylamide. Therefore, the diffusion coefficients should be correlated as 2.7 : 1, which is close to our experimental estimate (2.3 : 1).

Table 2. Displacement of the front boundary of DNA fragments with a length of 27 nucleotides in gels made with different concentrations of acrylamide

Time, h	Movement of the border of the front oligonucleotides, mm (coefficient of variation, %)	
	7% gel ($n = 3$)	10% gel ($n = 2$)
2	0.74 ± 0.06 (9%)	–
14	2.6 ± 0.05 (2%)	1.8 ± 0.4 (24%)
18.5	2.9 ± 0.2 (6%)	2.0 ± 0.4 (19%)
24	3.4 ± 0.2 (5%)	2.3 ± 0.5 (24%)
37	–	2.8 ± 0.6 (22%)
46	–	2.8 ± 0.6 (23%)
68.5	–	3.6 ± 0.5 (13%)
143	–	4.7 ± 0.4 (9%)

2.3. Movement of the front of labeled oligonucleotides in gels

Experimental values for fluorescently labeled synthetic single-stranded DNA fragments with a length of 27 nucleotides obtained in gels of different densities are presented in Table 2. The shorter fragment corresponds to an inertia diameter of 10.2 nm, so it is possible to use an estimate of the diffusion coefficient based on the diffusion distance (1) and an on analysis of the applicability of the spherical particle approximation (2). The estimate for the alternative method (3) is unacceptable, since the requirement defined for the ratio δ/L is fulfilled already for the second time frame (14 h) (the upper limit is exceeded).

An estimate of the tangent of the angle of inclination of the straight line for a 7% gel, plotted using all 12 experimental points in double logarithmic coordinates $(\ln(\delta); \ln(t))$, gives the result 0.63 ± 0.02 (coefficient of determination 0.986). This estimate is close to the theoretical value of 0.5. At the same time, measurements corresponding to the moment of time 2 h in case of conversion of coordinates according to the Huber concept can be considered as risk points where measurements can change estimates of linear trend parameters. However, unlike unbalance points, there is no categorical recommendation to exclude them. The Huber parameter h_j in these points is 0.319. According to the status of the risk points, this value should be in the range

from 0.2 to 0.5 [15]. Points with parameters less than 0.2 are safe, i.e. recommended for constructing approximating dependencies.

The values h_j are determined based on the measurement points by the expression (4), namely the logarithm x_j of the measurement times:

$$h_j = \frac{S_2 - 2S_1 x_j + S_0 x_j^2}{S_0 S_2 - S_1^2}, \quad (4)$$

where

$$S_k = \sum_{i=1}^{n} (x_i)^k, \quad k = 0, 1, 2.$$

Another linear approximation can be constructed by eliminating the measurement in these points. The estimation of the angular coefficient for this approximation gives the result 0.43 ± 0.06 (the coefficient of determination is also quite high, namely 0.876).

The minimum value for estimating the diffusion coefficient through the diffusion distance for 2 h, is $3.40 \cdot 10^{-11}$ m^2/s, while the average estimate of the diffusion coefficient through the diffusion distance δ for all other measurements gives the value of $(6.56 \pm 0.55) \cdot 10^{-11}$ m^2/s. However, in the first case, the impact of the error of determination of the initial moment of the diffusion time is possible with an excessively short a duration of observation of the front and the impact of boundary is possible in the second case.

The estimate δ was 1.78, 2.26 and 2.82 mm, respectively, in denser 10% gels for time counts 14, 24 and 41.5 h. The last estimate is the averaging over counts 37 and 46 h. Linearization of the dependence $\delta(t)$ in double logarithmic coordinates gives the tangent of the angle of inclination of the straight line (exponent of the degree of power function) slightly less than 0.5, namely 0.42 ± 0.02. The obtained dependence has high reliability, since the coefficient of determination exceeds 0.994.

The total estimate for all measured values corresponds to $(2.8 \pm 0.9) \cdot 10^{-11}$ m^2/s $(n = 14)$. If the diffusion coefficient is calculated only for the first point, for which most likely there are no boundary effects, then the value estimated based on the diffusion distance is $3.35 \cdot 10^{-11}$ m^2/s. The corresponding diameter of inertia is 9.7 nm, which is very close to the spherical Stokes-Einstein approximation. However, there is a certain „deceleration“ of the front boundary. The calculation of the diffusion coefficient over long times for large values δ demonstrates a decrease of the estimated value of the diffusion coefficient to $(2.5-2.6) \cdot 10^{-11}$ m^2/s at times of 46 and 68.5 h.

In natural coordinates (δ, t), the dependence is approximated by two linear sections with the „splice“ point (inflection of dependence) of $t = 36$ h with δ of about 2.8 mm. This may indicate that the nature of the dependence $\delta(t)$ changes with a long duration of the experiment and the result of the study is affected by an additional process, possibly associated with a decrease of the concentration of labeled fragments in the border zone.

The estimated values of the diffusion coefficient of oligonucleotides (according to the power dependence) change by the same 2.3 times as for DNA amplicons (see above) with an increase of the concentration of acrylamide from 7 to 10%.

Conclusion

The effect of acrylamide concentration on the diffusion of amplicons (DNA fragments obtained as a result of analysis) in a gel was studied in this paper. The following calculation methods were considered for estimation of the diffusion coefficients: 1) according to the power dependence based on the Einstein-Smoluchowski formula (at the same time, the conformance of obtained data with the hypothesis of diffusion displacement was verified according to the square root law); 2) according to the velocity of the boundary of the constant concentration [14], if the necessary conditions were met $(0.4 \leq \delta/L \leq 0.9)$; 3) in the approximation of a spherical particle according to the Stokes-Einstein formula (with an estimate of the equivalent diameter of inertia). The values of the diffusion coefficient obtained by the first and second methods (for amplicons in a denser 10% gel) are comparable. The second method can be recommended for the study of dense gels in which small diffusion movements are recorded. The model of equivalent sphere can be used to estimate the diffusion coefficient of short DNA fragments with a length of 27 nucleotides, however, there is a strong discrepancy in estimates for amplicons with a length of 103 bp in a 10% gel. Therefore, the third method can only be used for very approximate estimates.

An experimental power function assessment shows that the estimated diffusion values change 2.3 times both for amplicons with a length of 103 bp, and for DNA fragments with a length of 27 nucleotides with an increase of the concentration of acrylamide from 7 to 10%. This also correlates well with data calculated from the size of colonies formed by amplicons of similar length [2].

The proposed relatively simple method for estimating the diffusion coefficient can be used to predict the results of expected changes in colony sizes when controlling gel properties (by increasing the concentration of acrylamide). This will allow developing the topology of the reaction chambers of the microfluidic chip (and hence the geometric dimensions of the gel) depending on the task: miniaturization of the size of the reaction chambers, increasing the dynamic range of the method, optimizing the size of the resulting colonies, etc.

Conflict of interest

The authors state that they have no conflict of interest.

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Translated by A.Akhtyamov