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Agarose gel structure using atomic force microscopy: Gel concentration and ionic strength effects

Agarose gels have been studied by atomic force microscopy (AFM). The experiments were especially designed to work in aqueous conditions, allowing direct observation of the "unperturbed" gel without invasive treatment. AFM images clearly show strong dependence of pore diameter and its distribution on ionic strength of the solvent. As the ionic strength increases, the distribution becomes broader and the position of its maximum shifts toward higher values. The evolution of the distribution curves indicates that gels become more homogeneous with decreasing Tris-borate-EDTA (TBE) buffer concentration. An empirical law of the mean pore diameter as a function of the ionic strength is established. In agreement with our previous work we found that, for a given ionic strength, the pore diameter increases when the agarose concentration decreases and that the wide pore diameter distribution narrows as the gel concentration increases.

1 Introduction

The determination of the pore diameter in gel structures is of interest in various domains such as permeation and transport phenomena of biological macromolecules. The results from a variety of studies on gel structure by permeation [1], nuclear magnetic resonance [2], light scattering measurements [3], freeze-fracture and electron microscopy [4–6], have either supported or have been interpreted in terms of the presence of heterogeneities in the network. Although these studies constitute a valuable approach to the description of the gel structure, to date there no quantitative experimental study concerning in particular the pore diameter and its distribution in its native state. This is due to the experimental difficulty associated with the distortion of the structure during the experiment or to the destruction of the structure due to the formation of ice crystals during quenching in the case of, *e.g.*, freeze-etched electron microscopy experiments. All these methods are invasive and/or use a model to deduce the pore diameter. The experiments using atomic force microscopy (AFM) were specifically designed to work under aqueous conditions and allow direct observation of the "unperturbed" gel without invasive treatment. Many parameters, such as polymer concentration, ionic strength of the buffer, charge of the polymer, cooling speed, *etc.*, can influence the final structure of agarose gels. Among the parameters, two are easily adjustable by the experimentalist: the gel concentration and the ionic strength of the solvent. Recently we directly measured the pore diameter of agarose gel using AFM [7]. At a concentration of Tris-borate-EDTA (TBE) buffer equal to 0.01 M, we demonstrated that the pore diameter depends on the gel concentration following the scaling law $c^{-0.6}$ and that the gels present a wide pore diameter distribution except at a concentration of the order of 3%, where the distribution appears more homogeneous. Additional estimations of the pore diameter, made at lower concentrations (0.7% and 1%) than in [7] is presented in this paper. Working with a "liquid" cell in the AFM apparatus, filled with the same electrophoretic buffer, allows the direct comparison of the results with the pore diameters deduced from electrophoretic mobilities measured by fluorescence recovery after photobleaching [8] and using the biased reptation model [9–11]. The other parameter, which may influence the pore diameters, is the ionic strength of the solvent. Analyzing freeze-fractured surfaces, a pronounced difference between gels formed in the presence or absence of salt was observed [5]. Gels prepared in the absence of salt had larger free spaces, a result corroborated by measuring higher DNA electrophoretic mobilities in gels prepared in the absence of salt. This is important, because it could explain the disagreement encountered in literature regarding the pore diameter of the gel. Values given in literature for the pore diameter of a 1% agarose gel vary between 180 nm and 280 nm. Consequently, the composition of the electrophoresis buffer used during gel preparation may affect the mobility and resolution of DNA run during gel electrophoresis. The aim of this paper is to complete previous work on the influence of the agarose concentration on gel pore diameter and to better define the concept of "heterogeneity" in dependence on ionic strength by directly visualizing the gel structure using the AFM technique.

2 Materials and methods

2.1 Agarose and gel casting

Agarose was purchased as an electrophoretic grade (EEO : 0.08) from ICN (Lot No. 59898, Orsay, France). The agarose was dissolved in TBE buffer by boiling for a few minutes. The evaporated water was replaced to adjust the concentration to its initial value. The solution was then kept at 50°C for 1 h to ensure equilibrium. The gel was poured between two plates, allowed to gel, and kept for 30 min prior to AFM measurements. Images of gels, cast either in an open frame, or between two plates, or of cross-sectioned gels, were previously found to show no significant difference in average pore diameter

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Abbreviations: AFM, atomic force microscopy; **TBE,** Tris-borate-EDTA

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and its deviation. Gel casting between two plates for AFM has two advantages: (i) It allows us to use exactly the same protocol to prepare gels for our electrophoresis experiments. Our electrophoretic cell, inserted in the FRAP setup, is custom-built. It consists of two rectangular microscope plates, glued together with 1 mm spacers along three sides. Two platinum electrodes are positioned within a distance of 7.3 cm. (ii) The gel thickness (1 mm) is better controlled than in open frames. In addition to 1% gels, 0.7% agarose gels in 0.01 M TBE were also studied.

2.2 AFM

Relief domains were explored using the Nanoscope III commercial AFM (Digital Instruments) in the constant-force mode at 0.2×10^{-9} Newton. The large-scan type of scan head was used (130×130 μm^2 scan range). Calibration was performed with rulings for lateral distances, and ball bearings for vertical distances. All experiments were performed with a gel surrounded by a solution (fresh TBE) using a standard liquid cell at 21°C. The main problem in imaging soft gels with AFM is the relatively high force exerted by the cantiliver tip on the sample, which can be destructive. The images were obtained using the microscope feedback loop, which moves samples up and down, in order to maintain the force, applied by the tip, at a constant value. The force was minimized in order to avoid gel surface damage. Working in a liquid phase overcomes capillary forces between the tip and the gel, preventing water evaporation from the surface. Among the cantilevers provided by Digital Instruments, we had the choice of springs of rectangular or triangular geometries, of different lengths (100 or 200 μm) and of different widths (10 or 20 μm), and thus of different stiffnesses (from 0.1 to 0.58 N/m). The much longer springs were more flexible (and should allow a more sensitive surface scan). However, they were also sensitive to vibrations and consequently it was necessary to apply important forces on the gel surface to use them with a favorable signal-to-noise ratio; this, however resulted in more damage to the gel surface. To compromise, we used triangular springs, because this geometry allowed us to minimize the lateral friction forces. Thus, the springs with a high degree of stiffness were used preferentially. All the images presented here were obtained with a cantilever of spring constant 0.58 N/m. The images were processed by flattening to remove background noise. Around one hundred measurements of the pore diameter were performed on each image, with a special cursor on the screen activated by the software of the Nanoscope III (Fig. 1). Note the circular shape of the domains and their size polydiversity. The images exhibit a fine pore structure, similar to a sponge or crumb of bread, among clear (agarose bundles) and dark domains (pores) which reflect the roughness of the surface. The images are composed of 512×512 pixels, and they are presented in shades of grey corresponding to the variation of the measured signal, and thus the thickness. The distribution of the pore diameter displays different widths depending on the ionic strength or the agarose concentration. In order to show the significance of this distribution, we provided some statistics. A typical histogram, for the gel at 1 M of TBE, derived

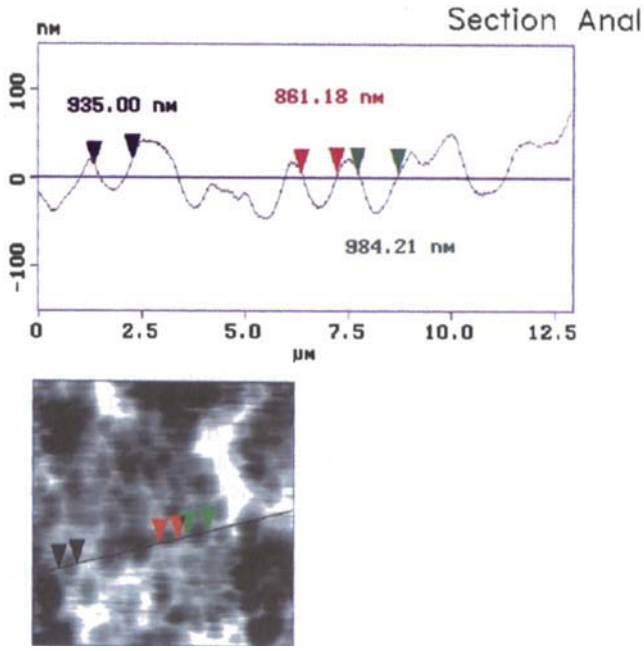

Figure 1. Example of pore diameter measurements using the cursor on the screen.

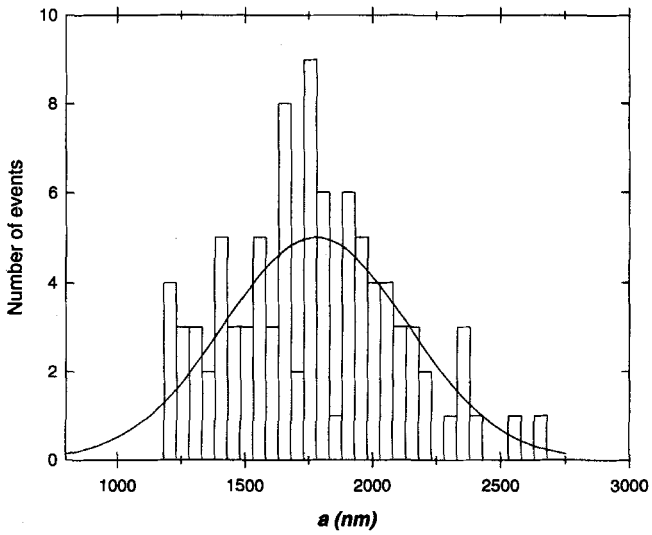

Figure 2. Pore diameter distribution for 1% agarose gel and 1 M TBE concentrations.

from one hundred pore diameter measurements, is plotted in Fig. 2. The solid line corresponds to a Gaussian distribution. The method to measure the mobilities and the theoretical background from which we deduced the pore diameter are described in detail in [8].

3 Results and discussion

3.1 Average pore diameter as a function of agarose concentration

In the initial reptation models, the chain was replaced by a series of N blobs of mean square end-to-end distance equal to a^2, where a is the tube diameter. The electrophoretic mobility is given by [9–11]

Table 1: $\mu_O/\mu_{E\to O}$, number of pores occupying N and pore diameter a for different agarose concentrations compared to values estimated from AFM images in 0.01 M TBE.

C(%)	0.5	0.7	1	1.5	2	3	4	5
$\mu_O/\mu_{E\to O}$	7.5±0.2	13.2±0.7	20.7±1.1	35±3	54±8			
N	2.5	4.4	6.9	11.7	18			
a(nm) from $\mu_{E\to O}$	810	610	490	370	300			
$<a>$ (nm) from AFM		530±185	509±90		364±84	289±66	243±46	201±36

$$\mu = <h_x^2>/(Na)^2 = \mu_0\left[\frac{1}{3N} + f(E)\right] \quad (1)$$

where μ_0 is the length independent mobility of DNA in solution, $<h_x^2>$ the mean square component of the end-to-end distance in the direction, x, of the applied field, E. By extrapolating the mobilities at vanishing low fields, absolute values of the pore diameter can be calculated from

$$\mu = \mu_{E\to O} = \mu_O\ (1/3N) \quad (2)$$

from which the pore diameter a can be calculated since, assuming Gaussian statistics in the absence of field,

$$<h^2> = Na^2 = N_O\ (2P)^2 = L\ 2P \quad (3)$$

where $<h^2>$ is the mean square of the end-to-end distance, N_O the number of Kuhn segments of the chain of length $2P$ (P=50 nm), and L its contour length. We used the λDNA fragment (48500 bp; base pair length: 3.4 Å) in electrophoresis experiments because it occupies a sufficiently high number of pores to satisfy the hypothesis of biased reptation. Average pore diameters obtained from our values of $\mu_{E\to O}$ and μ_O and from AFM are listed in Table 1. There is no error bar for a values from mobilities, because the data are themselves an average size, "viewed" by the chain during the migration.

There was no complete overlap between determination from mobilities and from AFM. This is due to the fact that, on the one hand, it is difficult for the DNA to enter the gel at high concentrations (to perform electrophoresis experiments) and on the other hand the quality

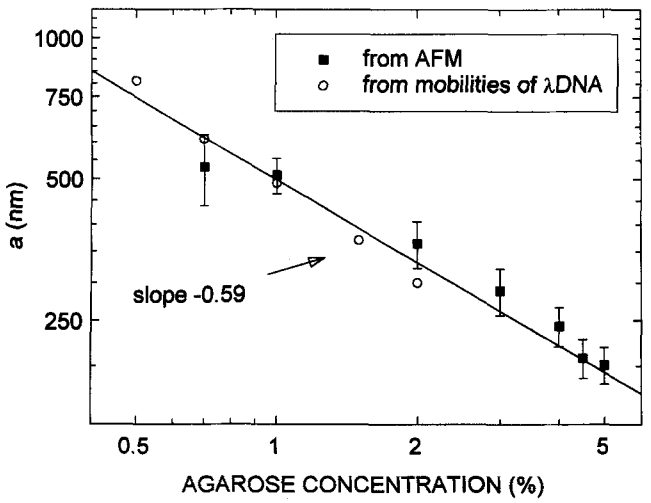

Figure 3. Variation of a from AFM images and deduced from mobility measurements as a function of agarose concentration; 0.01 M TBE.

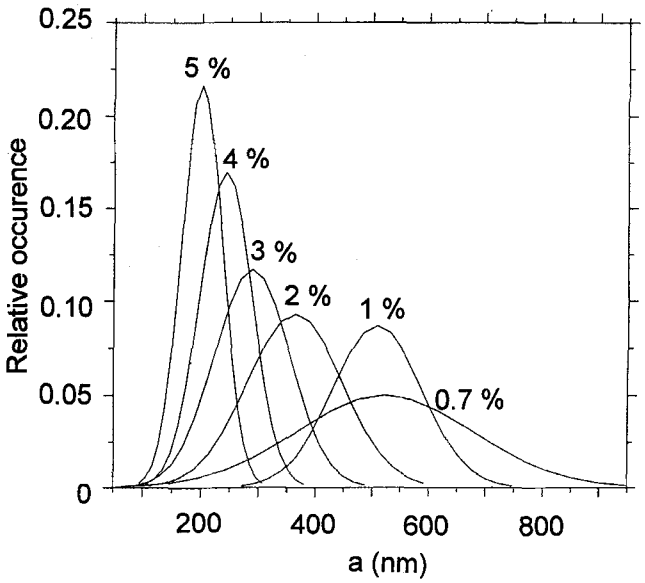

Figure 4. Evolution of the pore diameter distribution as a function of agarose concentration; 0.01 M TBE.

of AFM images is not adequate when the gel becomes too soft at very low concentrations ($\lesssim$ 0.5%). All a values are plotted as a function of the gel concentration in Fig. 3. Data determined from the model and from the AFM images are in good agreement. Obviously, there are very small pores that we cannot measure on AFM images but these small pores are also not "seen" by the reptating DNA during gel electrophoresis. The apparent pore diameters determined here are at least a factor of two greater than those determined by Slater *et al.* [10]. The mobilities measured by conventional electrophoresis in our agarose gels gave the same results [8]. The reasons remain unclear. Electrophoretic experiments (used to evaluate the pore size) have been reported [10] in which the gel was cast at different ionic strengths than ours, with different DNA loading concentrations and with different agarose sources. We find a power law dependence $a \sim C^{-\gamma}$ where $\gamma \cong 0.6$. It lies between the prediction of the Ogston model for a random array of straight chains (1/2) and the value predicted by De Gennes for a network of flexible chains (3/4). Figure 4 confirms that the pore diameter distribution increases with decreasing gel concentration [7].

3.2 Average pore diameter as a function of ionic strength [I]

Figure 5 presents a large-scale view of the domain population obtained at the free surface of the gels at a concentration of 1% and salt concentration range of 1 M – 0.001 M. The evolution of the distribution curves for the

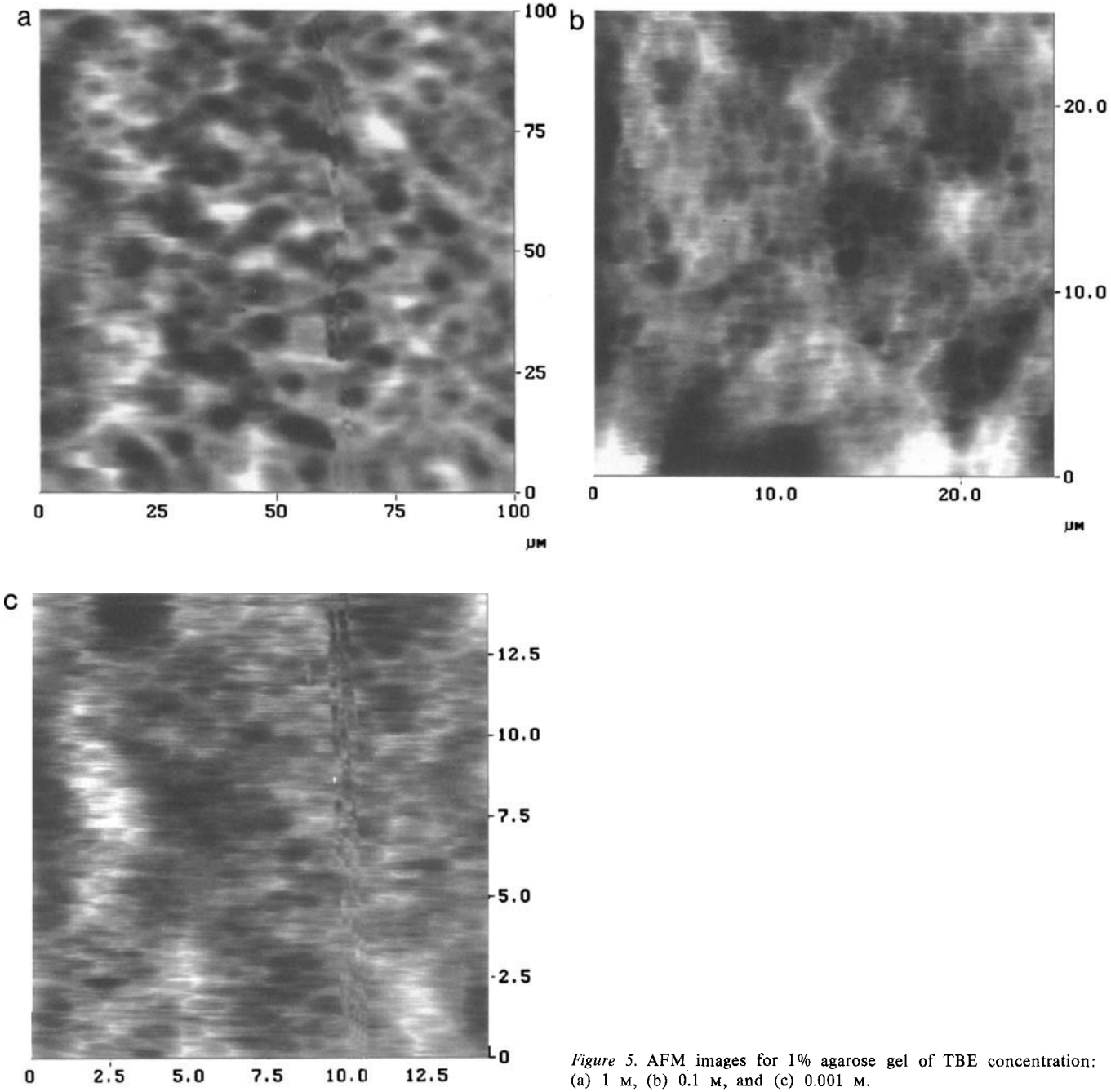

Figure 5. AFM images for 1% agarose gel of TBE concentration: (a) 1 M, (b) 0.1 M, and (c) 0.001 M.

different concentrations of TBE is shown in Fig. 6. We find that the gels become more homogeneous with decreasing ionic strength only with respect to the absolute values of the deviation. The percentage of pore size distribution seems to be remarkably invariant to the gel casting conditions (standard deviation $\cong 25 \pm 6\%$). The same is true for various agarose concentrations. This point will be investigated further when better statistics, using a greater number of pores (*e.g.*, using suitable software) is available. Valid speculations cannot begin with only one hundred manual measurements. As the ionic strength decreases, when the TBE concentration is changed from 1 M to 0.001 M, the maximum of the Gaussian fit (*i.e.*, the average pore diameter) increases and the distribution becomes broader. A large variation of the mean pore diameter is observed when the ionic strength is changed. Large pore diameters up to 3 μm in diameter were observed in gels of 1 M TBE concentration. The increase of pore diameter with the increasing ionic strength could be explained by the fact that the quality of the solvent decreases with increasing ionic strengths: the aggregation of the neutral agarose chains is favored in preference to the open cross-linked network, leaving larger free available domains. This was theoretically described by De Gennes [12] when changing the quality of the solvent of a polymer solution (for example by lowering the temperature). We have confirmed that making a 0.001 M gel and submerging it in 1 M solvent for 15 days does not affect the distribution and the average pore diameter. The fact that changing

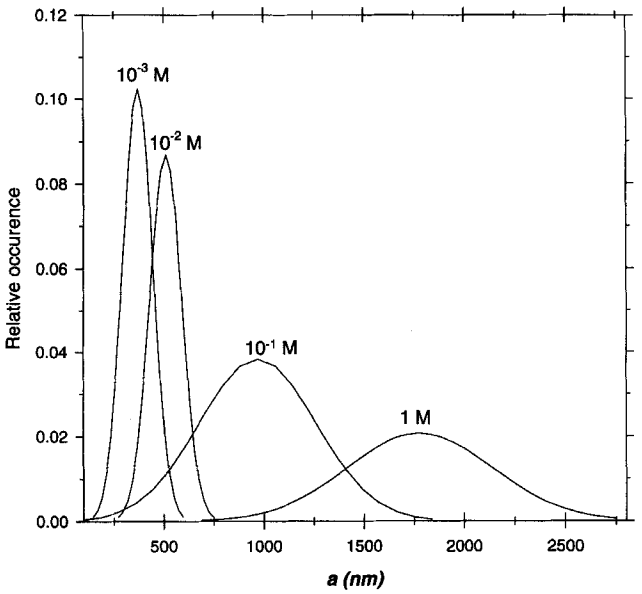

Figure 6. Evolution of the pore diameter distribution as a function of TBE concentration; 1% agarose

Table 2. Mean pore diameter as a function of TBE concentration in 1% agarose

TBE (M)	10^{-3}	10^{-2}	10^{-1}	1
<a> (nm)	370±78	509±87	966±350	1777±460

the running buffer does not affect the gel structure (*i.e.*, the pore size deduced from mobility measurements) has already been reported by Stellwagen [13]. We have noticed weaker mechanical properties when handling the 0.001 M gel. This suggests that the bundles constituting the gel contain less agarose helices, which consequently supports the existence of smaller pores and of a narrower distribution than for gels prepared at higher ionic strengths.

We have calculated the average diameter of the pore (denoted <a>) for each case and have directly obtained a reasonable estimation of the average pore diameter for each concentration in a model or probe-independent way. The average values are reported in Table 2. Figure 7 shows a log-log plot of the average diameter *versus* TBE concentration. The profile seems to be linear with a fitted slope equal to 0.25. This scaling law is valid only in the TBE concentration range of 1 M–0.001 M and with this particular agarose gel. A wide variety of agaroses is available for electrophoresis and it is anticipated that different agarose samples in the same range of TBE concentration will show the same behavior. These results are remarkable in that the opposite effects of using a higher TBE concentration and a higher gel concentration can be balanced to produce a gel that looks identical to one using standard conditions. The use of optimized TBE and agarose gel concentrations may facilitate migration of DNA fragments with resultant better separation. Using the laws determined above and the fact that there is no change in pore diameter when the gel is submersed after complete gelation in a buffer at a different ionic strength, both the agarose concentration and ionic strength of the gelation buffer may be utilized to optimize separation conditions. After gelation is complete, and prior to the actual separation, the casting buffer can be replaced by extensive soaking or an electrophoresis prerun with the running buffer of desired ionic strength.

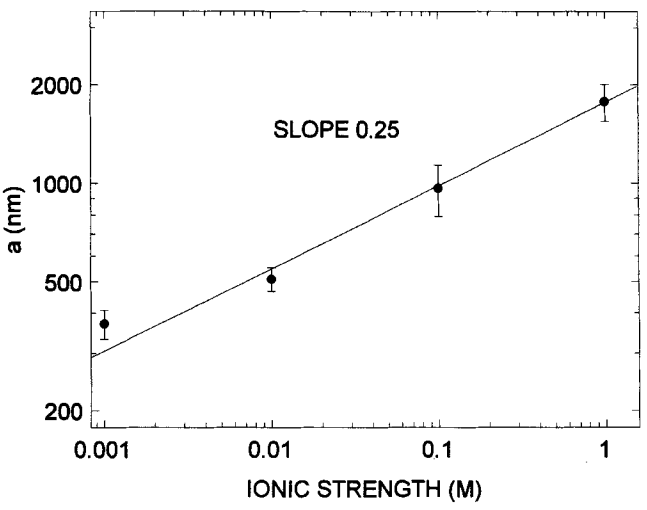

Figure 7. Pore diameter <a> as a function of the ionic strength; 1% agarose.

4 Concluding remarks

The average pore diameters of agarose gels can be estimated from AFM images in a cell especially designed to work under aqueous conditions allowing direct observation of the "unperturbed" gel without invasive treatment. We have shown that the average pore diameter and its distribution are affected by the ionic strength of the solvent and/or the gel concentration. The variation of the average pore diameter with the agarose concentration established from AFM images is compatible with that deduced from electrophoretic mobility measurements using reptation models.

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