Fast Two-Dimensional Bubble Analysis of Biopolymer Filamentous Networks Pore Size from Confocal Microscopy Thin Data Stacks

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ABSTRACT The average pore size $\xi_0$ of filamentous networks assembled from biological macromolecules is one of the most important physical parameters affecting their biological functions. Modern optical methods, such as confocal microscopy, can noninvasively image such networks, but extracting a quantitative estimate of $\xi_0$ is a nontrivial task. We present here a fast and simple method based on a two-dimensional bubble approach, which works by analyzing one by one the (thresholded) images of a series of three-dimensional thin data stacks. No skeletonization or reconstruction of the full geometry of the entire network is required. The method was validated by using many isotropic in silico generated networks of different structures, morphologies, and concentrations. For each type of network, the method provides accurate estimates (a few percent) of the average and the standard deviation of the three-dimensional distribution of the pore sizes, defined as the diameters of the largest spheres that can be fit into the pore zones of the entire gel volume. When applied to the analysis of real confocal microscopy images taken on fibrin gels, the method provides an estimate of $\xi_0$ consistent with results from elastic light scattering data.

INTRODUCTION

Filamentous networks are pervasive structural elements in biology, from the cell cytoskeleton to the hemostatic plug formed during blood coagulation. They are assembled by polymerization of biomacromolecular monomers, usually proteins, generating three-dimensional (3D) structures whose physical properties, such as fiber diameter, fiber length, and pore size, are directly linked to their mechanical properties and biological functions. Determining these properties is therefore important, and a wide variety of techniques have been developed over the years, going from microscopy (e.g. (1–3)) and scattering methods (e.g. (4–6)) to rheological and transport techniques (e.g. (2,7,8)).

Among noninvasive techniques, confocal microscopy has emerged as one of the most prominent, having the distinctive advantages of allowing the direct examination of the samples in real space, under near-native conditions, and often also as a function of time. However, due to the convoluted information and the random characteristics of most biological networks, extracting physical parameters from confocal microscopy data is not a straightforward task. Therefore, the search for robust and fast methods, keeping pace with the recent phenomenal technological advances in instrumentation and data acquisition (e.g. (9–12)), is an active field of research, often leading to the development of proprietary and expensive commercial software (13). A state of the art debate on the developments of new methods and software tools for quantitative biomedical imaging can be found in (14), and references therein.

Among the physical parameters characterizing the structure of biopolymeric filamentous networks, the average pore or mesh size is undoubtedly one of the most relevant, because it affects both their rheology and the diffusive transport phenomena (see (2,7,8)). The estimate of the average network pore size from 3D stacks of confocal images can be performed following different approaches and algorithms, some developed in two-dimensions (2D), others in 3D. Most of these methods, regardless of being 2D or 3D, require a threshold segmentation of the grayscale images to identify every pixel (or voxel) as belonging to a fiber ($n = 1$) or to the pore regions ($n = 0$). This procedure is quite critical, highly user-dependent, and leads to results that must be validated against other techniques or other known (or measured) parameters (see (15), and references therein).

2D methods are based either on the analysis of 2D projections of the entire stack (16), or, more commonly, on a one-by-one analysis of all the slices (17). When the slice sampling of the 3D stack is taken at random and the subsequent analysis is carried out on a statistical basis without any preliminary assumption, the method takes the name of (unbiased) stereology, a modern, still developing, interdisciplinary methodology that is concerned with the recovering of the 3D properties of a sample from its 2D sections (see for example (18), and references therein). Stereology can be profitably used in many bio- and natural-sizing applications where there is a necessity of estimating size, shape, and number of particulate objects, i.e., objects identified by a closed surface embedded in a given volume, such as cells, grains, holes, pores, more in general nonconnected (or partially connected) domains of any type. However, to the best of our knowledge, stereology is not suitable for...
the quantitative characterization of the 3D pore size of filamentous networks, where the pores occupy most of the sample volume and are fully interconnected.

3D methods are clearly more powerful, but also much more complex, time consuming, and quite demanding as far as concerns computational requirements. They usually require a skeletonization of a thick stack of thresholded images, in which the identification of individual fibers and the reconstruction of the gel scaffold are carried out in terms of the so-called fiber medial axis (19,20). Once the network is properly skeletonized, the detailed geometry of its architecture can be reconstructed by using sophisticated algorithms (21), leading to the recovery of pore size, fiber length, branching order, persistence length, and cross-linking spatial distributions.

Recently, a robust 3D method for determining the pore size of filamentous networks based on the use of the so-called (maximal) covering radius transform (CRT) (22,23) has been published (24). In this method, each voxel \( p \) of the stack belonging to a pore zone (called fluid phase in (24)), is assigned a value \( D_i(p) \) that corresponds to the radius of the largest sphere centered anywhere in the fluid phase, that is tangent to the fibers and covers the voxel \( p \). In this way, a distribution of covering radii can be retrieved, and its average value \( \langle D_i \rangle \) can be considered as a definition of the average gel pore size. The authors of (24) applied their method to the analysis of 3D stacks of confocal images taken on collagen networks grown from the polymerization of fluorescently labeled proteins. They showed that, by using the CRT in combination with the gel skeletonization based on the medial axis approach, it is possible to retrieve estimates of the gel pore size \( \langle D_i \rangle \) that are quite robust and reliable against user-dependent parameters employed in the analysis, and in particular against the threshold segmentation parameter.

As an alternative to truly 3D procedures, we present here a new, to our knowledge, 2D method for the determination of the gel pore size that, following a stereology-like approach, uses a simple homemade iterative algorithm for the analysis of thin stacks of randomly sampled thresholded 3D confocal images. The idea is to find, for each image of each stack, a set of nonoverlapping or slightly overlapping circles (called 2D-bubbles, see below) that can be optimally fit into the pore regions and produce their maximal coverage. The diameters \( D \) of these bubbles represent an estimate of the sizes of the pores in the different zones of the image. By repeating the procedure over all the selected slices of the stacks, a 2D diameters distribution \( P_{2D}(D) \) can be easily retrieved, and its average value \( \langle D \rangle_{2D} \) and standard deviation \( \sigma_{2D} \) determined.

The capability of our 2D bubble method of providing truly 3D information, i.e., the correspondence between \( \langle D \rangle_{2D} \) and \( \sigma_{2D} \) with respect the actual 3D parameters, was tested on many isotropic in silico-generated networks of different structures, morphologies, and concentrations, such as networks based on Voronoi tessellation or Delaunay triangulation (25,26), or networks mimicking the structural properties of biopolymer gels such as collagen gels (27) or fibrin gels (28). The 2D analysis was carried out by slicing the 3D gel volume into a stack of sections at different heights, which represent the output of an (ideal) confocal microscope. The final result of this analysis, i.e., the recovered \( P_{2D}(D) \) distribution of the 2D bubble diameters was compared with the corresponding \( P_{3D}(D) \) distribution that was retrieved in 3D by using a homemade semianalytical iterative algorithm similar to the one used for the 2D. \( P_{3D}(D) \) describes the distribution of the diameters of the largest spheres that would be used for covering the pore zones of the network, and its average value \( \langle D \rangle_{3D} \) can therefore be taken as a good definition of the gel pore size (very similar to the one proposed in (24)). The comparison between the two distributions shows that, for each network, the \( 2D \rightarrow 3D \) conversion factors of the average and standard deviation of the pore size distributions, i.e., the ratios \( \langle D \rangle_{2D}/\langle D \rangle_{3D} \) and \( \sigma_{2D}/\sigma_{3D} \), are independent of gel concentration and fiber diameter, but slightly depend on network morphology. For all the networks tested in this study, the overall variation of \( \langle D \rangle_{2D}/\langle D \rangle_{3D} \) was \( \pm 18\% \), whereas for \( \sigma_{2D}/\sigma_{3D} \) was \( \pm 40\% \). However, when the analysis is restricted to a gel of known morphology, both ratios are much more stable with fluctuations of only a few percents.

The actual applicability of our method on real samples was tested on confocal images of fibrin gels, from which we were able to obtain estimates of the gel pore size reasonably consistent with the ones recovered in our previous studies (2–5) and revised in our recent work (28).

**MATERIALS AND METHODS**

**Generation of in silico networks**

Five 3D in silico networks of different structures and morphologies were generated according to 1), a standard Voronoi tessellation; 2), a standard Delaunay triangulation; 3), a simulated annealing Euclidean graph generation (EGG) algorithm for mimicking the structural and elastic properties of collagen gels (27); 4), a Delaunay designed algorithm for producing photonic band gap (PBG) materials characterized by complete large band gaps in the optical frequency range (25,26); 5), a new homemade algorithm for the reconstruction of the static structural properties of fibrin gels (28).

The protocols for generating all the different networks, as well as the characterization of their geometrical properties, are described in the Supporting Material, Appendix A.

**3D-bubble analysis of gel pore size**

The 3D characterization of the gel pore size was carried out by using a homemade program, which allows to recover the distribution of the largest diameters \( D \) of the spheres (that from now on will be called bubbles for the reasons indicated below) that can be optimally fit into the pore zones of the gel and produce their maximum filling. The program works by using a pseudoanalytical iterative algorithm, which is based on the knowledge of the geometrical coordinates of the endpoints of all the

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fibers constituting the gel. For each bubble, the iterative algorithm works as follows:

a) a seeding point $C_0$ belonging to the pore region is generated at random.

b) the distances between $C_0$ and all the fibers (represented by segments passing for the fibers axis and ending at the fiber endpoints) are computed and sorted in increasing order, so that the four nearest distinct neighbor points belonging to four different fibers can be found.

c) the bubble passing for these four points is found analytically and its center $C_1$ and diameter $D_1$ are determined.

d) the new center point $C_1$ is accepted as part of the iterative procedure if it falls inside the tetrahedron defined by the four points laying on the bubble surface or, equivalently, if the four points define a surface larger than an hemisphere. This condition ensures that the bubble is effectively bounded or wrapped by the four fibers (not simply tangent to them) and, therefore, can be considered as a good representative of the pore associated to that zone because it provides its maximum coverage. If this condition is not fulfilled, the fourth point is rejected, the next nearest one is found, and steps c) and d) are repeated.

e) the new center point $C_1$ is considered as a new seeding point and steps a)–e) are iteratively repeated until convergence is reached, which occurs when the change in the coordinates of the bubble center is less than the required resolution, which was set to $10^{-3}$ of the average fiber length (typically 1 nm for our in silico gels). Convergence is typically attained within ~20 steps. Notice that the bubble found in this way is not tangent to four fibers, but to their medial axis or fiber endpoints. Thus, the bubble, which is effectively tangent to the gel’s four fibers, has the same center but a diameter reduced by the fiber diameter, which is $D_1^{-d}$.

By repeating the procedure outlined through points a1)–a5), one can find a set of bubbles that, because the seeding points are chosen at random, may contain duplicates or bubbles that are significantly overlapped. Thus, a discarding criterion is necessary: the bubbles are sorted according to their decreasing diameters and, starting from the largest one, each bubble is sequentially added to a set of covering bubbles (i.e., the bubbles used for covering the pore zones) only if it is not significantly overlapped to all others previously selected, otherwise it is discarded. Two bubbles of diameters $D_1$ and $D_2$ are considered to be significantly overlapped if the center of the smaller bubble lies inside the larger bubble. This condition happens when the distance between the bubbles centers $\Delta_{12}$ is smaller than the maximum between $R_1 = D_1/2$ and $R_2 = D_2/2$, i.e., if $\Delta_{12} < \max(R_1, R_2)$. Geometrically, this condition ensures that the contact angle $\theta$ between two bubbles ranges from zero (tangent bubbles) to a maximum value that occurs when $\Delta_{12} = \max(R_1, R_2)$, i.e., when the center of the smaller bubble lies on the surface of the larger bubble. In the latter case, $\theta$ ranges between 90° ($D_1 < D_2$) and 120° ($D_1 = D_2$). In general, two any nonsignificantly overlapping bubbles will have a contact angle <120°, regardless of their diameters $D_1$ and $D_2$. Thus, our overlapping/nonoverlapping condition, which was set empirically without a rigorous scientific justification, recalls closely what happens in real merging bubbles, and this is the reason why we decided to use the term bubbles for indicating the spheres used for filling the pore regions.

The including/discarding procedure leads to a coverage volume in which, as the number of included bubbles increases, the addition of new bubbles becomes less and less likely because they tend to be more and more overlapped to the old ones. Thus, because the time required for adding new bubbles becomes increasingly longer (and eventually diverges), the procedure must be stopped at a given point. We decided to adopt as a stopping criterion the attainment of a target filling ratio, which was set to a value of 0.5. This threshold was set empirically on the basis of a tradeoff between processing times, statistics, and accuracy. We checked that, above this value, the bubble diameter distribution $P_{3D}(D)$ does not change significantly, with the average bubble diameter $\langle D \rangle_{3D}$ and standard deviation $\sigma_{3D}$ varying <−1% with respect to their asymptotic (number of seeding points $\to \infty$) values. A detailed discussion and estimate of the errors introduced by setting a filling ratio threshold equal to 0.5 is reported in the Supporting Material, Appendix B.

An example of the overall bubble covering obtained with our method applied to an in silico gel generated with the method of (28), is shown in Fig. 1 a and, for a single bubble, is detailed in Fig. 1 b, where one can see three of the four contact points (red small dots) between the bubble and the surrounding fibers.

The correct functioning of our method was checked against known geometries such as simple randomly oriented gratings. A more general test was carried out by comparing our method with the well-known (maximal) CRT technique (22,23). The results of these comparisons are reported in the Supporting Material, Appendix B.

2D-bubble analysis of the gel pore size

The 3D analysis described previously represents an efficient method for estimating the gel average pore size, but requires the knowledge of all the fibers coordinates. Such information is clearly available for a synthetic gel, but not easily recoverable for real gels, which are typically investigated with confocal microscopy and therefore described in terms stacks of 2D

![Image](https://example.com/figure1.png)

FIGURE 1 3D bubble method applied to an in silico fibrin gel. (a) the red spheres represent the largest 3D bubbles (see text) that can be optimally fit in the pore zones of the gel and produce their maximum filling. (b) zoom of a single sphere touching four different fibers.
images representing slices of the gel volume at different heights. Based on the results of the previous section, we wondered what kind of information we could retrieve about the gel pore size if we applied the same bubble analysis to 2D images of the gel.

Thus, to simulate the output of an (ideal) confocal microscope, we sliced the 3D gel volume into a stack of \( N_z \) images, each made of \( N_x \times N_y \) pixels. Typically, our in silico gels were represented as a stack of 512 (or 1024) images, each made of \( 512 \times 512 \) (or \( 1024 \times 1024 \)) pixels, with a \( 50 \times 50 \times 50 \) nm or \( 25 \times 25 \times 25 \) nm cubic voxel. Because the fibers are randomly oriented cylindrical segments of diameter \( d \), their horizontal sections are randomly oriented ellipses characterized by the same short axis (equal to \( d \)) and by a different long axis whose length and orientation depend, respectively, on the incident and azimuthal angles between the fiber axis and the normal to the horizontal plane. An example of a 2D binary image corresponding to a horizontal section of a typical in silico gel is illustrated in Fig. 2a, where the ellipses represent the fibers \( (n = 1) \), the white zones are the pores \( (n = 0) \), and the circles filling the spaces between the fibers are the result of our 2D-bubble analysis. Indeed, such analysis consists in finding, for each image of the stack, the circles (that, in analogy with what was done for the 3D case, will be called bubbles) of maximum diameter \( D \) that can be optimally fit (see below) into the 2D pore zones and produce the maximum 2D coverage of the image. As shown in Fig. 2a, this coverage leads to the formation of bubbles that are tangent to the first three nearest fibers and can be partially overlapped. This procedure (developed by using the graphical routines available in LabView 2010) works with an algorithm similar to the one described for the 3D case, but it is entirely numeric because the fibers are pixilated and it is not necessary to know their coordinates. The only requirement is to have binary images as the one shown in Fig. 2a. The iterative algorithm that governs this 2D-bubble analysis is described in Fig. 2b and works as follows:

a1) a seeding point \( C_0 \) belonging to the pore region is generated at random.
a2) the distances between \( C_0 \) and the fibers (represented by the ellipses) are found and sorted in increasing order, so that the three nearest distinct neighbor points belonging to the borders of three different fibers are determined.
a3) the bubble passing for these three points is found and its center \( C_1 \) and diameter \( D_1 \) are determined (green dotted bubble in Fig. 2b).
a4) the point \( C_0 \) is moved in the direction of \( C_1 \) by an amount equal to a fraction \( \beta < 1 \) of the distance between \( C_0 \) and \( C_1 \).
a5) the new point is accepted as part of the iterative procedure if among its three nearest neighbor fibers there is at least one belonging to the triplet of fibers found for the point \( C_0 \). If this condition is not fulfilled, it means that the point \( C_0 \) has been moved too far away and cast into a pore region completely different from the starting one. Thus, the procedure through steps a4) and a5) is repeated again with a smaller moving step \( (\beta^2, \beta^3, \text{and so on}) \), until the previous condition is fulfilled.
a6) the new center \( C_1 \) is considered as a new seeding point and steps a1)–a5) are iteratively repeated until convergence is reached, which occurs when the coordinates of the bubble center do not change (within the pixel resolution) between one iteration and the next one.
a7) the final acceptance of the bubble is subjected to the condition that the triangle defined by the three points tangent to the bubble circumference is acute, implying that the bubble center falls inside the triangle. This condition ensures that the bubble is effectively bounded or wrapped by the three fibers, and not simply tangent to them. Thus, the bubble is a good representative of the pore associated to that zone because it provides its maximum coverage. Conversely, when this condition fails (blue dashed bubble in Fig. 2b), the fiber corresponding to the vertex with the obtuse angle (fiber n.2 in Fig. 2b) is removed from the image, the center \( C_1 \) is considered as a new seeding point, and the procedure restarts again from step a2).

The convergence rate increases with increasing \( \beta \), but if \( \beta \) is too high the procedure may become unstable and produce results that may slightly depend on \( \beta \). We found that for \( 0.3 \leq \beta \leq 0.7 \) the results are quite reproducible (<1% differences in average diameters) and convergence is typically attained in ~3–10 steps \((\beta = 0.5)\). On a standard PC (Intel Quad-core-i7 3.07 GHz, 8 Gb RAM) the analysis of a \( 512 \times 512 \) pixel image, takes about ~100–1000 ms per bubble. This time depends mainly on the density (surface fraction) of the fibers in the image, which determines the angular resolution with which the angular scan of step a2) must be carried out. Typically, for an image with a fiber surface fraction in the range \(-10^{-3} \text{ to } 10^{-4} \), an angular resolution of 0.1° is sufficient.

Once a set of bubbles has been found, the method goes through an including/discarding procedure similar to the one described for the 3D case: the bubbles are sorted according to their decreasing diameters and, starting from the largest one, each bubble is added to the set of the already selected covering bubbles only if it is not significantly overlapped to all of them, otherwise it is discarded. The overlapping criterion is equal to the 3D case: when the center of the smaller bubble lies inside the larger bubble two bubbles are considered to be overlapped. This criterion leads to contact angles <120°, from which the term bubbles was maintained also for the 2D case.

As for the 3D case, a tradeoff between processing times, statistics, and accuracy was adopted, and the coverage procedure was considered to be completed when the filling ratio of the covered zones (fibers included) reaches a value of 0.7. This threshold guarantees that, above this value, the bubble diameter distribution \( P_{2D}(D) \) does not change significantly, with the average bubble diameter \( \langle D \rangle_{2D} \) and standard deviation \( \sigma_{2D} \) varying <5% with respect to their asymptotic (number of seeding points \( \to \infty \)) values. A detailed discussion and estimate of the (systematic) errors introduced by setting a filling ratio threshold equal to 0.7 is reported in the Supporting Material, Appendix B.
Confocal microscopy of fibrin gels

Fibrin gels were polymerized under physiological conditions from solutions of fibrinogen (FG) and thrombin (Th), at two Th/FG molar ratio concentrations (1:100 and 1:1800). In both cases FG was labeled with Alexa Fluor 488 and its final concentration was 0.5 mg/ml. For a detailed description of sample preparation and labeling see the Supporting Material, Appendix D.

Fully formed gels were studied using an Olympus Fluoview 500 confocal microscope (Olympus Biosystems, Hamburg, Germany), equipped with a 60×, NA 1.40 oil immersion objective (PlanApo 00/0.17, Olympus) and excited with 488 nm laser light. For such a system, due to aberrations introduced by the oil-glass-water interfaces, the point spread function (PSF) of the microscope is highly dependent on the depth z inside the sample and becomes more and more elongated as z increases (29). Thus, our confocal data were taken by acquiring several thin stacks of images located in different zones of the sample, but at the same depth. Each stack was composed of 15 grayscale images of 1024 × 1024 pixels (pixel size d, = 207 nm), digitized with a 16 bit resolution, taken at a dz spacing of 100 nm starting from an initial depth of z1 = 14 μm and ending at z2 = 16 μm. The average depth of 15 μm inside the sample was the minimum depth at which we did not observe any anisotropy effects in the fibers orientation due to interactions with the cell window. Under these working conditions, the microscope PSF allows a resolution of ~200 nm laterally and 600 nm longitudinally (29). Note that the slightly undersampling along the lateral direction was for maximizing the field of view (~210 μm), without losing any capability of detecting individual fibers, whose diameter is expected to be ~200 nm (28). This tradeoff is unavoidable whenever confocal microscopy is used for imaging fibrous networks with pore sizes ~1–2 orders of magnitude larger than fiber diameters.

RESULTS AND DISCUSSION

In this section, we compare the diameter distributions recovered with the 2D and 3D bubble methods described in the previous section on a set of in silico gels characterized by different structures and morphologies (see Appendix A). In particular, we will compare the average diameters and standard deviations that are recovered with the two methods and see whether it is possible to establish a simple relationship between them. Our analysis will be described in detail for the case of in silico fibrin gels, for which experimental results will be also presented. Results regarding other network topologies will be here summarized, whereas their detailed description is deferred to the Supporting Material, Appendix A.

a) Pore size of in silico fibrin gels

These networks were generated according to the method described in (28), where they were showed to reproduce quite accurately the structure of real fibrin gels grown under quasiphysiological conditions. A detailed description of the algorithm used for generating these networks is reported in the Material and Methods section and in the Supporting Material, Appendix A of (28). Gels were generated at different volume fractions φ with the same fiber diameter d = 200 nm (set A) and different fiber diameters varying between 150 ≤ d ≤ 250 nm (set B). They are characterized by quite different fiber length distributions (lower φ, longer fibers, see Fig. S2 of (28)), but they exhibit the same geometrical properties, as illustrated in three panels of Fig. S3 of (28): 1), the angle distribution between fibers

<table>
<thead>
<tr>
<th>Network type</th>
<th>⟨α⟩ ± σα (degs.)</th>
<th>⟨k⟩ ± σk</th>
<th>σL (μm)</th>
<th>σD2D (μm)</th>
<th>σD3D (μm)</th>
<th>⟨D⟩2D (μm)</th>
<th>⟨D⟩3D (μm)</th>
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</thead>
<tbody>
<tr>
<td>Fibrin gels</td>
<td>102 ± 37</td>
<td>4.0 ± 0.9</td>
<td>0.36</td>
<td>0.36</td>
<td>0.28</td>
<td>1.16 ± 0.02</td>
<td>1.28 ± 0.02</td>
</tr>
<tr>
<td>Voronoi</td>
<td>111 ± 36</td>
<td>4.0 ± 0.0</td>
<td>0.73</td>
<td>0.33</td>
<td>0.19</td>
<td>1.00 ± 0.02</td>
<td>1.74 ± 0.03</td>
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<tr>
<td>Delaunay</td>
<td>93 ± 37</td>
<td>15.5 ± 3.3</td>
<td>0.32</td>
<td>0.50</td>
<td>0.34</td>
<td>1.14 ± 0.03</td>
<td>1.47 ± 0.04</td>
</tr>
<tr>
<td>EGG collagen</td>
<td>110 ± 32</td>
<td>3.4 ± 0.7</td>
<td>0.60</td>
<td>0.44</td>
<td>0.46</td>
<td>1.36 ± 0.01</td>
<td>0.96 ± 0.01</td>
</tr>
<tr>
<td>PBG</td>
<td>106 ± 27</td>
<td>4.0 ± 0.0</td>
<td>0.25</td>
<td>0.32</td>
<td>0.34</td>
<td>1.17 ± 0.02</td>
<td>0.94 ± 0.02</td>
</tr>
</tbody>
</table>

Note: standard deviations reported in cols. a and b refer to the distribution widths, whereas standard deviations reported in cols. f and g refer to statistical fluctuations evaluated over networks of different volume fractions φ.

*Average ± standard deviation of the angles between fibers;

*Average ± standard deviation of the branching order between nodal points connecting the fibers;

*Relative standard deviation of the fiber lengths;

*Relative standard deviation of the 2D bubble diameter distribution;

*Relative standard deviation of the 3D bubble diameter distribution;

*Ratio between the average diameters recovered with the 2D and 3D methods (independent of the network volume fraction).

*Ratio between the relative standard deviations of diameters recovered with the 2D and 3D methods (independent of the network volume fraction).
linked at the same nodal points is fairly broad, skewed toward small angles, with an average angle \( \langle \alpha \rangle = 102 \pm 37^\circ \); 2), the branching order distribution is characterized by an average value \( \langle k \rangle = 4.0 \pm 0.9 \); 3), the rescaled fiber length distribution is a smooth bell-shaped curve with a relative standard deviation \( \sigma_f(L) \approx 0.36 \).

The diameters distribution of gels of set A, obtained with our 2D and 3D bubble methods, are shown in Fig. 3, by an average value. It is worth noticing that, when the fibers have the same average diameters \( \langle D \rangle_{2D} \) and \( \langle D \rangle_{3D} \) decrease, with the 2D ones being (at the same \( \phi \)) always larger than the corresponding 3D ones. All the 2D (or 3D) curves appear to be very similar in shape and if we rescale them by \( \langle D \rangle_{2D} \) and \( \langle D \rangle_{3D} \) we obtain two single master curves, as shown in Fig. 3, b and d, respectively. These master curves exhibit similar shapes with slightly different relative standard deviations, namely \( \sigma_{2D}/\langle D \rangle_{2D} \approx 0.36 \) and \( \sigma_{3D}/\langle D \rangle_{3D} \approx 0.28 \). A quite similar behavior was observed for the gels of set B, whose rescaled distributions 2D and 3D distributions superimposed rather nicely to the ones reported in Fig. 3, b and d, respectively (data not shown).

A quantitative analysis of the data of Fig. 3 is shown in Fig. 4, where the behaviors of \( \langle D \rangle_{2D} \) (corrected for a 5% systematic error equal for all concentrations, see the Supporting Material, Appendix B) and \( \langle D \rangle_{3D} \) are reported as a function of \( \phi \) for both set A (circles) and set B (triangles). As one can notice, the behaviors of \( \langle D \rangle_{2D} \) and \( \langle D \rangle_{3D} \) are quite similar and consistent with two power-law decays, \( \langle D \rangle_{2D} \sim \langle D \rangle_{3D} \sim \phi^{-\gamma} \), with the exponents \( \gamma_A \approx 0.55 \) and \( \gamma_B \approx 0.37 \). Incidentally, it is worth noticing that, when the fibers have the same diameters as in the case of set A, the value of \( \gamma_A \) can be related to the mass fractal dimension \( D_m \) that characterizes the gel structure \( (4,5,27) \) by the relation \( \langle D \rangle_{3D} \sim \phi^{-1/(3-D_m)} \).

In this case the equivalent fractal dimension would be \( D_m \approx 1.2 \), a value consistent with both experimental and simulated data as shown in Fig. 9 a of (28).

The ratios \( \langle D \rangle_{2D}/\langle D \rangle_{3D} \) is shown in Fig. 4 b as a function of \( \phi \). The error bars refer, for each concentration, to averages performed over 20 independent images of area \( 51.2 \times 51.2 \, \mu m^2 \) (1024 x 1024 pixels) or 80 independent images of area \( 25.6 \times 25.6 \, \mu m^2 \) (512 x 512 pixels). As expected, because the overall averaged area is the same for all the concentrations and the number of bubbles/area is higher at higher \( \phi \), at higher concentrations the error bars decrease. However, even for the lowest concentrations, the uncertainty is of the order of a few percent.

In conclusion, for these networks the ratio \( \langle D \rangle_{2D}/\langle D \rangle_{3D} \approx 1.16 \pm 0.02 \) is nearly constant over the entire \( \phi \) range, showing that one can estimate the 3D average bubble diameter of fibrin gels from a measure of the 2D average diameter. A similar analysis carried out on the relative standard deviations of the two distributions (data not reported) shows that the ratio \( \sigma_{2D}/\langle D \rangle_{2D}/\sigma_{3D}/\langle D \rangle_{3D} \approx 1.28 \) is nearly constant as well. Thus, a measure of the first two moments of the 2D distribution, i.e., \( \langle D \rangle_{2D} \) and \( \sigma_{2D} \), allows us to retrieve accurately both \( \langle D \rangle_{3D} \) and \( \sigma_{3D} \).

b) 2D-3D correspondence between bubble diameters

The geometrical properties of all the networks studied in this work and the correspondence between the results found with our 2D and 3D bubble methods are summarized in Table 1.

The table shows that there is no evident correlation between the geometrical parameters of the different networks (2nd to 4th columns) and the distributions of the bubble diameters, either 2D or 3D. In particular, the relative standard deviations of fiber length (4th column), 2D diameter (5th column), and 3D diameter (6th column) distributions appear to change widely from network to network, but remain constant inside each morphology, independently of network concentration. The same behavior occurs for the factors.

![Figure 3](image-url)
describing the 2D → 3D conversion, i.e., the ratios \( \langle D \rangle_{2D} / \langle D \rangle_{3D} \) (7th column) and \( \sigma_{2D} / \langle D \rangle_{2D} / \sigma_{3D} / \langle D \rangle_{3D} \) (8th column). Thus, we conclude from Table 1 that we can estimate the actual 3D values of \( \langle D \rangle_{3D} \) and \( \sigma_{3D} \), from a measure of \( \langle D \rangle_{2D} \) and \( \sigma_{2D} \), provided that network morphology is known and that the correct 2D-3D conversion factors (7th and 8th columns) are used. In any case, if no information on network morphology is available, rough estimates of \( \langle D \rangle_{3D} \) and \( \sigma_{3D} \) can still be retrieved by using, as a conversion factors, the central values of the ranges spanned by the figures appearing in the 7th and 8th columns, i.e., the values 1.18 and 1.34. In that case, the maximum errors on \( \langle D \rangle_{3D} \) and \( \sigma_{3D} \) would be, respectively, \( \pm 18\% \) and \( \pm 40\% \).

For comparison, we also report in Table 2 the results of our 2D and 3D bubble methods carried out on randomly oriented regular gratings generated from a simple cubic lattice (row 1), a body-centered cubic (BCC) lattice (row 2), a Voronoi tessellation with seeding points laid on a face-centered cubic (FCC) lattice (row 3), a Voronoi tessellation with seeding points on a BCC lattice (row 4), and a diamond lattice (row 5). All the gratings, which have been generated starting from a cubic cell of side \( a \) (2nd column) have mono or bimodal branching orders \( k \) (3rd column) and fiber lengths (4th column).

The results of our analysis shows that, for all the gratings, the 3D-bubble diameter \( D_{3D} \) is monodisperse (6th column), whereas the corresponding 2D diameters are highly polydisperse (5th column). As for all the networks of Table 1, the ratio \( \langle D \rangle_{2D} \langle D \rangle_{3D} \) (7th column) differs fairly little from grating to grating, thus allowing us to estimate \( \langle D \rangle_{3D} \) with higher accuracy than in the case of random networks. In the last column (8th column), the wide variation of the ratio \( \langle D \rangle_{2D} / L \) shows that also in the case of regular grating, there is no evident correlation between geometrical parameters and distributions of the bubble diameters, either 2D or 3D.

c) Pore size of real fibrin gels

We finally report an example of our 2D bubble method applied to the analysis of real confocal data taken on two fibrin gels prepared at the same FG concentration \( c_{F} = 0.5 \text{ mg/ml} \), but with different Thr concentrations. In the first sample (gel A) the ratio between the Thr/FG concentrations was 1:100, whereas in the second sample (gel B) the ratio was 1:1800. The raw confocal images of these gels (see Fig. 5, a and b) show that their morphology is qualitatively similar (straight fibers joined at some nodal points whose branching order is \( \sim 3-4 \)), but characterized by different pore sizes because the average distance between fibers is clearly smaller in gel A than in gel B. This feature is consistent with what is known from the literature, where a lower concentration of Thr leads to larger pores and thicker fibers (see (30), and references therein). Notice that, because of mass conservation, gels with smaller pores are made of thinner fibers, but this difference is not appreciable from the figure because of the convolution with the microscope PSF.

The confocal data of the two gels were preprocessed by following the procedure outlined in the Supporting Material.
1.16 (see previous section) are ones (obtained by dividing the 2D values by the factor (28)); the thresholding parameter was set to 1/4 of the expected diameter (~200 nm, see Fig. 9).

The 2D-bubble diameter distributions, reported in Fig. 5, were retrieved with the 2D bubble method by following the preprocessing procedure outlined previously, we must point out that such a procedure can be very subtle because the spatial resolution of the microscope is comparable with the expected fiber diameter (~200 nm) and with the pixel size (~207 nm). In these cases the fibers orthogonal to the horizontal plane occupy ~1 pixel, and it is troublesome to distinguish them from noise. Thus, the convolution with the narrow Gaussian distribution (σ = 0.25 pixel) is quite critical and may lead to filtering out real fibers with the consequence of overestimating $\langle D_A \rangle_{2D}$, which is probably the reason why our 2D bubble method retrieves a gel pore size higher than the ELS data. We also checked the dependence of the results on the value assigned to the thresholding parameter γ. Changing γ from 0.30 to 0.40, we retrieved values for $\langle D_A \rangle_{2D}$ varying from ~10.0 to ~10.5 μm, consistent with what was reported in Appendix E.

CONCLUSIONS

In this work, we have described a simple 2D method for the determination of the 3D average pore size and standard deviation of isotropic biological filamentous networks from confocal microscopy data. The method works by analyzing one by one the slices of a 3D data stack, or, better, of a few slices of several thin stacks, without any necessity of skeletonization or reconstruction of the 3D geometry of the entire network. The only requirement is a thresholding segmentation of the 2D images. The method works by finding, for each image, a set of slightly overlapping circles (called 2D-bubbles because their overlapping features are similar to what happens in real merging bubbles) that can be used for covering the maximum area of the pores zones. Each bubble is found by using a homemade algorithm that optimizes the bubble center and diameter, until the largest bubble located anywhere in a pore region and tangent to the three nearest neighbors fibers is found.

Although the methods work in 2D, it can provide truly 3D information, provided that some general assumptions (gel isotropy) are fulfilled. By using many isotropic in silico-generated networks of different morphologies and concentrations such as standard Voronoi or Delaunay networks, or networks reproducing structural properties of PBG material (25,26), collagen gels (27), or fibrin gels (28), we have shown that the 2D → 3D conversion factors of the average and standard deviation of the 2D and 3D pore size distributions, i.e., the ratios $(D)_{2D}/(D)_{3D}$ and $(σ_{2D}^2/(D)_{2D})/(σ_{3D}^2/(D)_{3D})$ depend moderately on network type.
maximum variation over all the networks reported in Table
1, are $\pm 18\%$ for $\langle D \rangle_{2D}/\langle D \rangle_{3D}$ and $\pm 40\%$ for $(\langle \sigma \rangle_{2D}/
\langle \sigma \rangle_{3D})_{2D}/\langle \sigma \rangle_{3D}$. (Table 1) and are almost constant (few
percents), independently of gel concentration and fiber
diameter, when networks of the same morphology are
considered. It should be mentioned, however, that, when
networks exhibiting some anisotropy (for example, elon-
gated pores aligned along one direction) are investigated,
the recovery of the $P_{2D}(D)$ distribution may become highly
inaccurate. In that case the slicing of the 3D structure could
be done not only at random $z$ positions, but also with random
orientations. Nevertheless, the interpretation of the results is
always troublesome because, as known from stereology
(18), this method always provides nonnegligible overesti-
mates of the (round) average pore size.

A further relevant advantage of our 2D method over stan-
dard 3D methods is that it can work with thin stacks.
Because there is no necessity of 3D reconstruction, the
bubble statistics can be accumulated by analyzing indepen-
dent images taken over different portions of the sample,
possibly at the same depth $z$ below the coverslip. This
is particularly important for two reasons: i), biological
samples get easily bleached when imaged over thick stacks,
most of all when high-resolution (and high-power) laser
illuminations, such as in a stimulated emission depletion
confocal microscope, are used; ii), biological samples are
commonly imaged (for economic reasons) with oil immers-
ion objectives, implying that the microscope PSF is highly
dependent on $z$, and the 3D reconstruction of thick stacks
may become troublesome.

The definition of gel pore size adopted in this work is
similar to the one proposed in (24), where the so-called
(maximal) CRT (22,23) has been used. In both cases, the
gel pore size is identified as the average diameter of the
largest spheres that can be located in the pore zones.
However, besides being respectively 2D and 3D, these two
methods differ because they retrieve different kinds of diam-
eter distributions. Our 2D method recovers a number $P_{2D}(D)$
distribution because, due to the covering procedure, each
bubble diameter is counted only once during the average
computation. Conversely, the CRT method recovers a weight
(or volume) $P_{3D}(D)$ distribution because the statistics is per-
formed over all the voxels belonging to the pore regions.
A further, but probably marginal difference is that, due to the
covering filtering procedure, the CRT includes into the
statistics the diameters of spheres located in the proximity
of the fibers nodal points. These spheres are systematically
smaller than the largest spheres that one would fit in the
pores and are not present in our method.

The actual applicability of our method for the character-
ization of confocal images taken on real biopolymer net-
works was tested on fibrin gels, which were used in this
study as a model system for assessing its performances.
By comparing real and synthetic confocal images of fibrin
gels, effects due to the microscope finite resolution and pres-
ence of noise could be taken into account by convolving the
entire gel structure with the 3D PSF of a typical microscope and adding a realistic amount of statistical noise on each
2D image. This provided an optimization of the threshold
segmentation procedure, which allowed us to define a robust
and reliable protocol for data processing. When applied to
confocal images of real fibrin gels, the method provided
estimates of the gel pore size consistent with the ones recov-
ered in our previous studies (2–5) and revised in our recent
work (28).

In conclusion, we believe that our 2D bubble method
represents a useful tool for the characterization of isotropic
filamentous biological networks and in particular for the
determination of the average and standard deviation of their
3D pore size distribution. Further work is in progress for ex-
tending the method to networks with morphologies differ-
ent from the ones investigated in this work, such as networks
made of semiflexible fibers not modeled as straight cylind-
ers, or anisotropic networks.

**SUPPORTING MATERIAL**

Appendix, nine supplemental figures, and references (31–34) are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(13)00086-6.

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