Size and Density of Fibers in Fibrin and Other Filamentous Networks from Turbidimetry: Beyond a Revisited Carr–Hermans Method, Accounting for Fractality and Porosity

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ABSTRACT: The Carr–Hermans method [Macromolecules 1978, 11, 46–50], often used for determining the fibers diameter $d$ and density $\rho$ in fibrin or other filamentous networks from turbidity data, is found to be remarkably inaccurate when the system’s mass fractal dimension $D_m$ is $>1$. An expanded approach based on the knowledge of the system $D_m$ and pore size $\xi$, which can be accurately recovered from low-angle elastic light scattering data or estimated from confocal microscopy, is proposed. By fitting the turbidity data with a function obtained by numerically integrating the fibrin-optimized scattering form factor of a network of cylindrical elements, both $d$ and $\rho$ can be independently recovered. Numerical simulations were employed to validate the reliability and accuracy of the method, which is then applied to evolving fibrin gels data. More in general, this method is extendible to the analysis of other filamentous networks that can be represented as ensembles of cylindrical elements.

INTRODUCTION

Biopolymer filamentous networks and gels are ubiquitous and important structural elements in the living world, such as the cell cytoskeleton,1 the extracellular matrix,2 and the blood coagulation system.3 Their physiological and pathological (e.g., thrombosis4 and cancer metastasis5) relevant mechanical characteristics are strictly correlated to their physicochemical properties, which depend not only on the size and density of the fibers constituting the network but also on average bulk parameters such as the network pore size and its fractal morphology.6 The latter is associated with both the extent of fiber branching and entanglement, playing a crucial role in the network’s viscoelastic and rheological properties,7 which in turn determine its biofunctionality.8 Fractal dimension and porosity can be estimated rather easily by using various microscopy techniques, whereas the investigation of the fibers properties is more difficult, mainly because of dehydration issues in electron microscopy and lack of the required resolution in light microscopy. Although with the advent of super-resolution light microscopy techniques measuring diameters down to $\sim50$ nm and below is becoming possible, the use of specialized costly equipment and other issues, such as bleaching or lack of adequate time resolution, can be serious obstacles.9 Higher resolution scattering techniques such as small-angle X-ray scattering (SAXS) or small-angle neutron scattering (SANS) can be also employed, but they require complex setups, often within large specialized facilities.10

In 1978, Carr and Hermans proposed in this journal11 a simple method for determining, from the wavelength dependence of turbidity data, the mass/length ratio $\mu$ and the density $\rho$ (and thus the diameter $d$) of fibrin fibers resulting from the thrombin-induced polymerization of the blood coagulation protein fibrinogen. Requiring only a spectrophotometer, a standard staple in most laboratories, the Carr–Hermans (CH) method has been used for decades and is still being applied in a rather ubiquitous way, whenever there is a necessity of estimating the size and density of the fibers constituting a biopolymer filamentous network.12 The CH method is based on the assumption that the fibers of the network can be assimilated to a solution of noninteracting randomly oriented cylinders that are supposed to be very thin and infinitely long when compared with the wavelengths of the measurements. Such a sample corresponds to having a system with a mass fractal dimension $D_m = 1$. However, biopolymer filamentous networks are very far from these ideal conditions, being often characterized by fractal morphologies with $D_m > 1$, pore sizes $\xi$ not so large and fibers diameters not so thin with respect to the
wavelength. Thus, as it will be shown below, the application of the CH method to these networks may lead to remarkable errors in the determination of the fibers’ parameters.

In this work we followed a different approach, based on the knowledge of the parameters $\xi$ and $D_m$ and on their functional dependence in the scattering form factor of the network. We demonstrate that it is possible to fit the turbidity data by numerically integrating such a form factor and accurately recover both the diameter $d$ and the density $\rho$ of the fibers. Note that the parameters $\xi$ and $D_m$ have to be determined independently from the turbidity data by using either data provided by low-angle elastic light scattering (LAELS) or somewhat less accurate estimates given by other techniques such as, for example, confocal microscopy or rheometry.

We analyzed the specific, but relevant, case of a fibrin network whose form factor has been successful applied to the LAELS characterization of a variety of fibrin gels grown under quasi-physiological conditions. The validity of the method was tested by using numerical simulations and real data taken on a polymerizing solution forming a fibrin gel. To this purpose, we developed a LAELS setup coupled to a fiber-optic spectrophotometer, with the possibility of taking simultaneous scattering and turbidity measurements on the same network. The results indicate that when $\xi$ and $D_m$ can be determined independently (as in this case via the LAELS measurements), the turbidity fitting procedure developed in this work recovers accurately both the diameter $d$ and the density $\rho$ of the fibers. Moreover, this procedure can also be applied to time-resolved data, as shown here with the analysis the late stages in the formation of a fibrin network.

Finally, it should be pointed out that although the method proposed in this work has been devised and optimized for the specific case of fibrin gels, it is of much wider interest and can be applied to a large variety of biological, synthetic, or other geometrical filamentous networks that are describable as an assembly of cylindrical elements. Examples include collagen gels, photon band gap networks, and in silico networks based on Voronoi tessellation and Delaunay triangulation.

## THEORY

### Original Carr–Hermans Method

The original Carr–Hermans method is based on the light scattered by a collection of equal fibers randomly dispersed in a dilute solution. If the fibers are described as straight cylinders of length $L$, diameter $d$, density $\rho$, and mass/volume concentration $c$, the scattered intensity distribution is

$$ R(q) = Kc\mu P(q, L, d) $$

where $q = (4\pi/\lambda)\sin(\theta/2)$ is the scattering wavevector (being $\theta$ the scattering angle, $\lambda$ the light wavelength in vacuo, and $n$ the refractive index of the solvent), $K = (4\pi^2/N_A\lambda^4)n^2(dn/d\lambda)^2$ is the optical constant (being $dn/d\lambda$ the specific refractive index increment of the solute in that solvent and $N_A$ Avogadro’s number), $M = N_A(\pi/4)\rho dL$ is the fiber molecular weight, and $P(q, L, d)$ is the cylinder form factor normalized so that $P(q=0, L, d) = 1$. Note also that eq 1 is reported in absolute units, and $R(q)$, called Rayleigh ratio, represents the power scattered by the sample per unit solid angle, per unit incident power, and per unit length of the scattering volume and is customarily expressed in cm$^{-1}$.

For cylinders with a very large aspect ratio ($L \gg d$), the form factor can be expressed analytically as

$$ P(q, L, d) = P_{rod}(q, L)S_{sc}(q, d) $$

where $P_{rod}(q, L)$ and $S_{sc}(q, d)$ are respectively the form factor of a thin rod and its cross section:

$$ P_{rod}(q, L) = \frac{\sin(qL/2)}{qL/2} $$

$$ S_{sc}(q, d) = \frac{2J_1(qd/2)}{qd/2} $$

In eqs 3, $S_i(x)$ and $J_1(x)$ are the sine integral defined as $S_i(x) = \int_0^x \sin(t)/t \, dt$ and the first-order Bessel function, respectively.

The original CH method works on the assumption that the spectral range of the sample is a sample of dilute solution of thin ($d \ll \lambda$), straight, infinitely long ($L \gg \lambda$) fibers. The first assumption ensures that the argument of the Bessel function $qL/2 < (2\lambda/\pi)\sin(\theta/2)$, so that $J_1(qd/2)$ is small. The second assumption ensures that the form factor of the rod can be approximated to $P_{rod}(qL) = \pi/(qL)$. With these two approximations, critically discussed in the next section, eq 1 becomes

$$ R(q) = Kc\mu \pi 2q^2 \frac{1 - q^2d^2/16}{\lambda} $$

which can be conveniently rewritten as

$$ R(\lambda) = 8\pi \int_0^\lambda R(\theta)(1 - 2\sin^2\theta + 2\sin^4\theta) \, d\Omega $$

where $x = q/q_{max} = \sin(\theta/2)$. If we insert in eq 6 the Rayleigh ratio given by eq 4, we obtain the original Carr–Hermans result

$$ \tau(\lambda) = \frac{8\pi^3}{15N_A}n(dn/d\lambda)^2c\lambda^{-3} \left[ 1 - \frac{23}{77} \pi^2n^2d^2\lambda^{-2} \right] $$

Note that for very thin fibers ($d \ll \lambda$) the correction factor appearing in eq 7 (the second term inside the square brackets) can be neglected, and the turbidity scales as $\tau(\lambda) \sim \lambda^{-1}$. Thus, Carr and Hermans found convenient to use the reduced variables $x = \lambda^{-1}$, $y = c\tau^{-1}x^{-3}$, assume that $(23/77)\pi^2n^2d^2/\lambda^3 \ll 1$, and making the approximation $[1 - (23/77)\pi^2n^2d^2/\lambda^3]^{-1} = 1 + (23/77)\pi^2n^2d^2/\lambda^3$, they worked out eq 7 as

$$ y(x) = A\mu^{-1} \left[ 1 + \frac{23}{77} \pi^2n^2d^2x \right] $$

where $A = N_A[(8/15)\pi^4n^2(dn/d\lambda)^2]$. Equation 8 corresponds to the so-called Carr–Hermans plot (eq 6 in ref 11), in which
the $y(x)$ function scales linearly with $x$. The $x = 0$ intercept of the straight line gives the mass per unit length ratio $\mu$, while the slope allows to retrieve the diameter $d$, or equivalently the density $\rho$. It should be pointed out that the linear behavior predicted by eq 8 is actually observed only when the dispersion effects associated with the $\lambda$ dependence of $n$ and $dn/d\lambda$ are negligible and thus are considered constant. As shown in Supporting Information Appendix A, this approximation may lead to rather inaccurate estimates of $\mu$ and $d$, mostly when relatively small diameters ($d \leq 100$ nm) are considered. However, it is always possible to correct the CH plots for these effects and retrieve satisfactorily accurate values for $\mu$ and $d$ (see Supporting Information Appendix A).

**Deviations from the Carr–Hermans Method.** As mentioned above, the CH method works correctly only in the limit of very thin ($d \ll \lambda_0$) and infinitely long ($L \gg \lambda_0$) fibers. When these conditions are not met, we expect to observe substantial deviations from the linear behavior predicted by eq 8. Figure 1a shows four CH plots (symbols) of fibers with the same diameter $d = 50$ nm and density $\rho = 0.4$ g/cm$^3$, but different lengths $L = 5, 10, 30$, and 500 $\mu$m. The lines represent linear fits through the high $x$-data (solid symbols). The CH plots have been obtained from the exact $\tau(\lambda)$ curves, i.e., the ones computed by using eq 6, with $R(q)$ given by eqs 2 and 3. The $\lambda$-range was $0.3-1.0 \mu$m, and to simplify data interpretation, no dispersion effects were considered (constant $n$ and $dn/d\lambda$). As it is evident, the shorter the fiber, the larger is the upward curvature of the corresponding curve. It must be said that this effect is even more evident in Figure 1a because the fibers are very thin, making the deviations from linearity comparable with the dynamic range of the $y$ variable, which in this case is $\sim 5\%$. Nevertheless, all the data can be accurately fitted with a linear regression (solid lines), provided that only high $x$-data ranges are considered (solid symbols). This is shown in the residual plot of Figure 1b, where the deviations of all the data are of the same order of magnitude. Over these ranges, the fiber parameters were recovered with satisfactory accuracy, with maximum deviations of $\Delta\mu \sim -3\%$ and $\Delta d \sim -4\%$ for the case $L = 5 \mu$m.

Completely different types and extents of deviations are observed when the fibers diameter becomes comparable with the $\lambda$-range of the measurement. Figure 1c shows the CH plot for fibers with $L = 100 \mu$m, $\rho = 0.4$ g/cm$^3$, and $d$ varying between 50 and 300 nm. For the sake of clarity, the two upper data sets have been shifted vertically by arbitrary offsets so to rescale them on the same vertical axis. With the exception of the $d = 300$ nm data (see below), the lines passing through the points represent the best linear regressions of the low $x$-values data (solid symbols). For each diameter, the maximum $x$-value of the range was chosen so that all the residuals (Figure 1d) were within $\pm 1\%$. As one may notice, as $d$ becomes larger, the (downward) curvature of the data increases, and the range over which they can be accurately fitted with a linear regression becomes narrower. However, in spite of these differences, we were able to retrieve, for all of them, the fiber parameters with satisfactory accuracy, i.e., $\delta\mu \sim \pm 1\%$, $\delta d \sim \pm 1\%$ for $d = 50, 100$, and 150 nm and $\Delta\mu \sim -2\%$, $\delta d \sim -2\%$ for $d = 200$ nm. In the case of $d = 300$ nm, the linear fit was highly inadequate, and we fitted the low $x$-data with a second degree polynomial fit, obtaining accuracies of $\Delta\mu \sim -2\%$, $\delta d \sim +3\%$.

It is quite interesting to notice that the two upper data sets of Figure 1c ($d = 50$ and 100 nm) follow the linear behavior predicted by eq 8 even though the assumption ($d \ll \lambda_0$), which has been used both in eq 3b ($d \ll \lambda_1/(2\pi n) \sim 0.12\lambda_1 n = 1.33$) and eq 7 ($d \ll \lambda_1/(23/77)^{1/3} \sim 0.44\lambda_1$), is far from being fulfilled ($d/\lambda_1 \sim 0.17-0.3$). This behavior is not completely surprising because, similarly to what happens with the Zimm plots21 in the analysis of low $q$-vector elastic light scattering data, the two approximations used in eqs 4 and 7 tend to balance each other, and as a matter of fact, the linear behavior predicted by eq 8 persists well beyond its limits of applicability. Thus, using eq 8 is much more accurate than using eq 7 (data not shown). A discussion about this feature was also reported in ref 21 where the authors state (erroneously) that the use of eq 7 (corresponding to eq 5 of ref 21) is more accurate than using the original CH results, i.e., eq 8.

Finally, we investigate what happens in the opposite limit, when the fibers are very thick ($d \gg \lambda_0$). This is the so-called Porod regime,22 where the Rayleigh ratio depends on the density of the surface area, and for cylindrical fibers is given by

$$R(\theta) = \frac{KcM - \frac{32}{Ld^4}q}{q}$$

(9)

Unfortunately, this function cannot be directly integrated because of the $q^{-4}$ divergence in $q = 0$. However, this divergence is fictitious because eq 9 holds only for $qd \gg 1$, while for lower $q$ values it behaves accordingly to $R(q) \sim q^{-4}$. The crossover between the $q^{-1}$ and $q^{-4}$ behaviors occurs at $qd \sim (32/\pi)^{1/3} \sim 2.2$, and its actual shape depends on diameter polydispersity. By supposing to have a Lorentzian-type cross-over, such as for example $R(q) = Kc(M/L)/(q^2/32 + (\pi/\gamma))$, eq 9 can be numerically integrated and we obtain

$$\tau(\lambda) = 2\pi\rho n(dn/d\lambda)^2\gamma d\lambda^{-2}$$

(10)

where $\gamma$ is a numerical constant that in the limit of infinitely long fibers with monodisperse diameters is $\gamma \sim 8.41$. This is the value that we will use in the rest of this paragraph. By comparing eq 10 with eq 7, we notice a profound difference with what we found in the case of thin fibers: (i) for very thick fibers the turbidity is proportional to $pd$ (instead of $pd^2 \sim \mu$), and (ii) it decays as $\tau(\lambda) \sim \lambda^{-2}$ (instead of $\lambda^{-3}$). The
crossover between the $\lambda^{-2}$ and $\lambda^{-3}$ behaviors can be found by equating eq 7 with eq 10, giving

$$\lambda/n \approx 2.70d$$  \hspace{1cm} (11)$$

which, in water ($n = 1.33$), corresponds to $\lambda \sim 3.60d$. This crossover can be seen in Figure 2, in which the behavior of $\tau(\lambda)$ corresponding to infinitely long fibers is reported as a function of the ratio $\lambda/d$ on a log–log plot. The two different exponent values are indicated by the two straight lines, and one may notice that the crossover region, occurring around $\lambda/d \sim 3.60$ ($n = 1.33$), is fairly extended. Thus, if the $\lambda$-range of the measurement is not very large (as it usually happens), an apparent slope between $-2$ and $-3$ can be detected, making the data interpretation fairly troublesome, as it will be described in the next section.

As a final comment, we would like to point out that eq 8 is valid for a monodispersed solution of cylinders, i.e., cylinders with the same diameter $d$, density $\rho$, and length $L$. Polydispersity effects on $d$, $\rho$, and $L$ are discussed in Supporting Information Appendix B. Here we summarize only the main results stating that if the length distribution is statistically independent of the diameter and density distributions, the polydispersity in $L$ is irrelevant and the parameters recovered from the CH analysis depend only on the polydispersity on $d$ and $\rho$. In particular, only the molar weight-average mass per unit length $\langle \mu \rangle_w = N_p(\pi/4)(\rho d^2)\omega$ and the $z$-average square diameter $\langle d_z^2 \rangle$ can be recovered (see eq B.7 in Supporting Information Appendix B). Note that there is no possibility to retrieve also $\rho$, unless the density and diameter distributions are statistically independent and the “width” $\langle d_z^2 \rangle_w/(\langle d_z^2 \rangle_w)$ of the diameter distribution is known. In that case we can also recover $\langle \rho \rangle_w$ and $\langle d_z^2 \rangle_w$.

**Turbidity of a Fractal System.** In the previous sections, we have described and discussed the performance of the CH method when applied to long straight fibers, i.e., objects characterized by a mass fractal dimensions $D_w = 1$. In this section we generalize the method to the case of gels made of fibers that are fully interconnected and form a three-dimensional network characterized by a fractal morphology, with mass fractal dimensions $1 \leq D_w \leq 2$. We will specifically refer to the case of fibrin gels, for which the CH method was originally developed and is constantly applied, but the results illustrated below will have a general validity and could be also applied to other filamentous networks.

When examined with a confocal microscope (see Figure 3), a fibrin gel appears as a collection of straight fibers, not uniformly distributed in space, connected together at some branching points. Although each single fiber is quite straight and therefore behaves as an object with $D_w = 1$, when the overall system is considered the spatial arrangement of these fibers produces a structure with $D_w > 1$. We can model this complex structure by following the approach suggested originally in refs 13–15 and recently refined in refs 23,24, according to which the gel is a collection of densely packed, spatially correlated fractal blobs of size $\xi$, centered around the regions where the fiber concentration is higher. The blobs are placed at an average distance $\xi_0$ between their centers of mass, so that they overlap by a factor $\eta = \xi/\xi_0$. Each blob is made of an assembly of $k$ segments or “building blocks” which can be sketched as cylindrical objects with diameter $d$ and length $L$. These segments are bonded end-to-end so to form straight fibers, but they can also branch off at some bonding site, producing a fully interconnected ramified structure made of straight fibers of different lengths. As described in ref 23, the length of these fibers is fairly polydisperse, ranging between a minimum value of the order of $d$ and a maximum value of the order of $\xi$. According to this picture, the segment length represents therefore the minimum fiber length between two branching points, while a fiber is made of many segments joined together and can be much longer than the segment length $L$.

As shown in ref 23, the average distance $\xi_0$ between the blobs provides an accurate estimate of the gel pore size, i.e., the average size of the pores between fibers, which can be quantitatively defined as the average diameters of the largest spheres that can be accommodated between the gels’ fibers.24 However, it was also found that most of gel studied in ref 23 were characterized by an overlapping parameter close to unity, i.e., $\eta \sim 1-1.5$. Thus, since under the working conditions of this study ($\xi_0 \sim 5-10 \mu m$) the value of $\eta$ does not significantly affect the value of the turbidity (see the discussion at the end of Supporting Information Appendix C), we set $\eta = 1$, so that $\xi = \xi_0$ and the blob size $\xi$ can be interpreted as the gel pore size. In the rest of this article, $\xi$ will be called either “blob size” or “gel pore size”.

For a mass fractal system, the number of segments inside each blob scales as $k \approx (\xi/1)^{D_w}$, and therefore the blob molecular weight $M$ is given by the product of $k$ times the segment mass $m = (\rho \pi d^2 L)$, being $\rho$ the segment (or fiber) density. Following ref 23, we set $I = d$ and therefore

$$M \approx N_A \frac{\rho \pi}{4} \xi_0^2 \xi^{(3-D_w)}$$

(12)

Note that for $D_w = 1$ eq 12 provides the exact molecular weight of a stiff cylinder with $\xi = L$, i.e., $M = N_A(\pi/4)\rho d^2 L$. The scattering from such a system can be quantitatively described by a form factor, whose complete expression is rather complicated.

![Figure 3](image-url)
and we forward the reader to ref 23 and to Supporting Information Appendix C for a detailed description of this function. It is worth noticing that a similar derivation of the form factor that we originally developed for fibrin gels19 was used some years ago for the analysis of SANS data from cellulose networks.25,26

In the following, we will focus on the behavior of the fibrin form factor under the common situations in which the CH method is applied. When the blobs are very large \((q_0^2 \gg 1)\) and the fibers are very thin \((qd \ll 1)\), the Rayleigh ratio of a fibrin gel can be approximated to \(^{23}\)

\[
R(q) = Ke^{[\delta(D_m)]^{1/3}}Na_0^{2p/3} q^{-\delta(D_m)}
\]

(13)
or, in terms of the mass per unit length ratio of the fibers \(\mu\), to \(^{23}\)

\[
R(q) = Ke^{[\delta(D_m)]^{1/3}} \mu d^{1/3}q^{-\delta(D_m)} (1/d \ll q \ll 1/\xi)
\]

(14)

where \(\mu = N_q \pi d^2/4\) and \(\delta(D_m)\) is a dimensionless factor that for any given \(D_m\) depends also on the detailed morphology of the system. In the case of fibrin gels, with the help of the model described in ref 23 we found that \(\delta(D_m)\) assumes a sigmoid shape centered around \(D_m = 1.4\), from \(\delta(D_m) \approx \pi\) at \(D_m = 1\) to \(\delta(D_m) \sim 2.76\) at \(D_m = 2\). Notice that for \(D_m = 1\) eq 14 coincides with the first factor of eq 4. Equations 13 and 14 show that if \(d\) and \(\xi\) are quite different (as it usually occurs), \(R(q)\) decays over a large range of \(q\)'s as a power law with a decay exponent equal the fractal dimension \(D_m\).

If we now integrate angularly eqs 13 and 14, making use of eqs 5 and 6, we obtain

\[
\tau(\lambda) = 8\pi^4 \alpha(D_m) n^{2-n} \frac{d}{dc} \left( \frac{\rho d^{1/3-n} \lambda^{-2/3-n}/4}{\lambda^{1/3-n}/4} \right)
\]

(15)
or equivalently

\[
\tau(\lambda) = \frac{32\pi^3}{N_q} \alpha(D_m) n^{2-n} \frac{d}{dc} \left( \frac{\rho d^{1/3-n} \lambda^{-2/3-n}/4}{\lambda^{1/3-n}/4} \right)
\]

(16)

where

\[
\alpha(D_m) = \left[ \delta(D_m)^{1/3} \frac{1}{2 - D_m} - \frac{2}{4 - D_m} + \frac{2}{6 - D_m} \right]
\]

(17)
is a dimensionless quantity. Notice that for \(D_m = 1\), \(\delta \approx \pi\) and \(\alpha = 11/(60\pi)\), so that eq 16 reproduces exactly the first term of eq 7, which occurs when \(q \ll 1\). In general, the presence of the exponent \(\lambda^{1/(3-n)}\) makes the behavior of \(\tau(\lambda)\) significantly different from what is predicted by eq 7 \((\sim \lambda^{-3})\), and consequently the CH plots are expected to be curved also when the fibers are infinitesimally thin. Furthermore, the amplitude of \(\tau(\lambda)\) is also strongly dependent on \(D_m\) (mainly via the term \((\lambda d)^{3-n}\)), making a gel with higher \(D_m\) to be much more turbid. This is shown in Figure 4a, where \(\tau(\lambda)\) is plotted vs \(\lambda\) for gels with the same \(d = 50\) nm and \(\rho = 0.4\) g/cm\(^3\) (and therefore the same \(\mu\)), same concentration \(c = 0.1\) mg/mL and \(\xi = 100\) nm, but different \(D_m\) = 1.0, 1.2, 1.4, and 1.6. The corresponding CH plots (Figure 4b) show a linear behavior only for the case \(D_m = 1\) (upper straight line) and become progressively curved (downward) as \(D_m\) increases. Thus, it becomes very difficult to distinguish the reason why a CH plot is curved, either because of straight thick fibers (Figure 1c) or because of branched thin fibers (bottom curve in Figure 4b). Figure 4a reports on the following two behaviors for long thick cylinders with \(L = 100\) nm and \(d = 160\) nm (blue circles) and a fractal gel with \(d = 50\) nm, \(\xi = 100\) nm, and \(D_m = 1.5\) (red squares). Figure 5 shows an even more dramatic example of the ambiguity that turbidity data would exhibit. In panel (a) we report the \(R(q)\) behaviors for two very different systems, such as long thick cylinders with \(L = 100\) nm and \(d = 160\) nm (blue circles) and a fractal gel with \(d = 50\) nm, \(\xi = 100\) nm, and \(D_m = 1.5\) (red squares). The concentrations of the two samples have been arbitrarily adjusted so to have similar amplitudes for the two datasets. By numerically integrating the corresponding curves of panel (a) according to eq 6. Note that despite the quite different \(R(q)\) behaviors, the \(\tau(\lambda)\) curves are fairly superimposed, with power law decay characterized by the same slopes \(-2.5\). Thus, it is quite evident that turbidity data might be fairly misleading, and it is difficult extracting from them reliable information, even when the fibers are very thin. As a final comment, we would like to point out that eqs 13–16 refer to a gel with monodispersed blob size \(\xi\), density \(\rho\), and fiber diameter \(d\). Polydispersity effects on \(\xi\), \(\rho\), and \(d\) are discussed in Supporting Information Appendix C, where we show that eq 15 remains unchanged, provided that the term \(\rho d^{3-D_m}\) is replaced with \((\rho d^{3-D_m}/c)^{1/3}\). Thus, regardless of any polydispersity in \(\xi\), \(\tau(\lambda)\) provides information on a quantity that

![Figure 4](image-url)  
Figure 4. (a) A log–log plot of \(\tau(\lambda)\) for fibrin gels with the same \(d = 50\) nm, \(\rho = 0.4\) g/cm\(^3\), \(\xi = 100\) nm, but different \(D_m\) varying in the range 1.0–1.6, bottom to top. (b) Carr–Hermans plots corresponding to the data of panel (a); the maximum deviations from the linear behavior are found for the highest \(D_m\) data.

Furthermore, the zero intercepts of the various CH plots are quite different, implying quite different values for \(\mu\). As an example of the errors that can be made, we fitted the CH plot corresponding to the case \(D_m = 1.60\) with a linear regression (bottom straight line) passing through the high \(x\)-value data (solid circles), and we recovered the fibers parameters with huge errors, i.e., \(\delta\mu \sim +350\%\) and \(\delta d \sim +200\%\).
when $D_m \approx 1$, is similar to the weight-average mass per unit length ratio of the fibers ($\rho d^2 \eta$), exactly as it happens for straight cylinders (see Supporting Information Appendix B).

**Fitting the Turbulence Data: A New Data Analysis Approach.** To overcome the difficulties described above, we propose a new method for fitting the turbulence data, based on the knowledge of the gel pore size $\xi$ and mass fractal dimension $D_m$. The values of $\xi$ and $D_m$ can be estimated by using different techniques (such as confocal microscopy$^{16}$ and rheometry$^{17}$) or can be accurately recovered by using a low-angle elastic light scattering (LAELS) setup, as the one we previously described.$^{3,14}$

An example of synthetic LAELS and turbidity data corresponding to a fibrin gel at a fibrogen concentration $c = 0.5$ mg/mL, with $d = 200$ nm, $\rho = 0.4$ g/cm$^3$, $\xi = 10$ $\mu$m, $D_m = 1.3$, $\beta = 1$, and $\eta = 1$, is reported in panels (a) and (b) of Figure 6. The LAELS data have been generated by using the

![Figure 6. Synthetic LAELS (a) and turbidity (b) data generated for a fibrin gel at a fibrogen concentration $c = 0.5$ mg/mL, with $d = 200$ nm, $\rho = 0.4$ g/cm$^3$, $\xi = 10$ $\mu$m, and $D_m = 1.3$. A 3% RMS noise was added to both data sets. The solid lines are the best fitting of the data, carried out as described in the text. Panels (c) and (d) report the corresponding relative residuals of each fit.](Image)

$R(q)$ function given by eq C.1, whereas the turbidity data have been obtained by angularly integrating $R(q)$ as described in eqs 5 and 6. No dispersion effects were considered (constant $n$ and $dn/dc$) and a 3% rms noise was added to both data sets. Many different data sets corresponding to independent 3% rms noise configurations were analyzed for accumulating good statistics. The analysis, which was aimed at recovering only the parameters $\rho$, $d$, $\xi$, and $D_m$ (being $\beta = 1$ and $\eta = 1$ parameters that can be considered fixed to these values; see Supporting Information Appendix C), was carried in two steps: (i) We first fitted the LAELS data, from which only $\xi$ and $D_m$ were recovered very accurately (see second row of Table 1). Indeed, these are the only two parameters controlling the shape of $R(q)$, with $\xi$ being associated with the peak position and $D_m$ to the asymptotic slope (see the straight line in Figure 6a). Note that since the LAELS amplitude scales as $R(q) \sim \rho d (3-D_m)$ (see eqs 13 and 14), the parameters $\rho$ and $d$ are highly correlated, and it was not possible to recover them independently (see second row of Table 1). (ii) We then fitted the turbidity data by fixing $\xi$ and $D_m$ to the values found from the LAELS fitting and leaving $\rho$ and $d$ as floating parameters. In this way the values of $\rho$ and $d$ could be recovered with very high accuracies, $\sim 1\%$ (see third row of Table 1).

To check the reliability of this two-step procedure, we also carried out a global fitting, in which LAELS and turbidity data are fitted simultaneously; this means that the two data types are fitted with the two fitting functions described above, both controlled by the same floating parameters, namely $\rho$, $d$, $\xi$, and $D_m$. In this case, the $\chi^2$ minimization is carried out by taking into account the (weighted squared) deviations of each data set from its corresponding fitting function. The global and the two-step fitting procedures provide quite similar results: they fit the data with the same precision and recover all four parameters with quite similar accuracies and standard deviations, as shown in second to fourth rows of Table 1.

Importantly, we would like to point out that fitting the turbidity data alone, without any knowledge of $\xi$ and $D_m$, would lead to somewhat inaccurate and unreliable results. For example, if the data of Figure 6b were fitted with $\rho$, $d$, and $D_m$ as floating parameters, even with $\xi$ fixed to the correct value, the uncertainties on the retrieved parameters would be remarkably high. This occurs because, for the turbidity data, the three parameters $\rho$, $d$, and $D_m$ are all highly correlated. Indeed, the amplitude of $\tau(\lambda)$ scales as $\sim \rho d (3-D_m)$ whereas the slope of its power law decay is controlled by both $d$ and $D_m$. Thus, in spite of the fact that the data behavior can be accurately reproduced by the fitting function with no systematic residuals (such as in Figure 6d), the recovery of these parameters might be characterized by very large standard deviations, which in the case of $d$ and $D_m$ are even higher than the mismatching with the expected values (see fifth row of Table 1).

Finally, we estimated the errors made in the retrieval of $\rho$ and $d$ if the turbidity data are fitted with wrong values assigned to the fixed parameters $\xi$ and $D_m$. The results of this analysis, reported in Supporting Information Appendix D, show that for relatively large diameters ($d \geq 100$ nm) the accuracy in the recovery of $\rho$ and $d$ depends mainly on $D_m$ and is always $\sim \pm 10\%$, provided that $D_m$ is known with a $\pm 20\%$ error; on $\xi$ are less important, leading to accuracies that for typical fibrin gels with pore sizes $\xi \geq 5-10$ $\mu$m are always $\sim \pm 5-10\%$. Conversely, for small diameters ($d \leq 50$ nm), a correct value for $D_m$ is highly mandatory, but this can be recovered directly from the $\tau(\lambda)$ data. In the latter case, however, $\rho$ and $d$ cannot be recovered independently, but only the mass/length ratio $\mu$.

### EXPERIMENTAL SECTION

As discussed in the previous section, the measurement of the gel pore size $\xi$ and fractal dimension $D_m$ can be accurately performed by using the LAELS technique. Furthermore, with this technique not only the structure of the final aged gel but also the kinetics of the polymerization process can be studied.$^{3,14}$ Thus, carrying out simultaneously LAELS and turbidity time-resolved measurements would also provide a robust evaluation of the evolution of the density

**Table 1. Average and Standard Deviation Parameters $\rho$, $d$, $\xi$, and $D_m$ Recovered by Fitting Many Independent Sets of LAELS and Turbidity Data Corresponding to the Same Gel of Figure 6, with Added 3% RMS Statistical Noise**

<table>
<thead>
<tr>
<th>fitting*</th>
<th>$\xi$ ($\mu$m)</th>
<th>$D_m$</th>
<th>$\rho$ (g/cm$^3$)</th>
<th>$d$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>input</td>
<td>10.0</td>
<td>1.30</td>
<td>0.40</td>
<td>200</td>
</tr>
<tr>
<td>LAELS</td>
<td>10.0 ± 0.1</td>
<td>1.30 ± 0.02</td>
<td>$\rho d^{(3-D_m)} \sim (3.3 \pm 0.2) \times 10^3$</td>
<td></td>
</tr>
<tr>
<td>r(\lambda) &amp; 10.0 fix &amp; 1.30 fix</td>
<td>0.397 ± 0.02</td>
<td>202 ± 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>global</td>
<td>10.0 ± 0.1</td>
<td>1.30 ± 0.01</td>
<td>0.400 ± 0.01</td>
<td>200 ± 8</td>
</tr>
<tr>
<td>r(\lambda) &amp; 10.0 fix &amp; 1.44 ± 0.20</td>
<td>0.412 ± 0.03</td>
<td>177 ± 39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The data were fitted by adopting the different procedures described in the text.
ρ and diameter d of the growing fibers. To this aim, we have added to the previously described LAELS apparatus a turbidimeter made of two commercial fiber-optic miniature spectrophotometers. As shown in Figure 7a, the sample cell is alternatively illuminated, using two computer-controlled shutters, with a focused He–Ne laser beam (λ = 0.6328 µm) and a white light beam obtained by using a fiber-optic collimator. The LAELS detector, which is made of an array of annular photodiodes, is located on a half of the Fourier plane, i.e., the plane where the laser beam is focused. In this way, a mirror placed in the blind zone of the Fourier plane can reflect the white light transmitted by the sample and forward it onto a collecting lens that is coupled, via a second optical fiber, to the sample spectrophotometer. A second (reference) spectrophotometer is used for normalizing the turbidimetry data, similar to what is usually done in a standard two-arm photodiode setup. (b) Schematic diagram of the entire turbidimetry setup.

Figure 7. (a) Schematic diagram of the overall LAELS plus turbidimetry setup. (b) Schematic diagram of the entire turbidimetry setup.

RESULTS AND DISCUSSION

As previously reported, the growth kinetics of a fibrin gel formed under physical–chemical conditions similar to the ones utilized in this study is characterized by three regimes: a networking phase during which the scaffold of the network is initially formed, followed by a ripening phase where the network structure slightly changes and the fibers start to grow laterally, and finally by a thickening phase where the gel structure remains the same and the fibers become increasingly thicker (in terms of a growth of the mass/length ratio). An example with this behavior is shown in Figure 8, where the four data sets reported in panels (a) and (b) refer to LAELS and turbidimetry data taken at different times after addition of Thr to the FG solution. The time points were chosen after both LAELS and turbidity data started to rise above the background noise.

As one can notice in Figure 8, in this time range both LAELS and turbidity data exhibit a remarkable growth of their amplitudes, but with only slight changes of their shape distributions. In the case of LAELS data, the peak position moves slightly to the left of the q-range, suggesting a minor increase of the gel pore size, at the same time, the asymptotic slope of the power law decay changes from ∼1.2 to ∼1.4, suggesting an increase of the gel mass fractal dimension Dm. In the case of the turbidity data, the slopes of the power law decays change from ∼−2.9 to ∼−2.7, meaning that the network is either increasing the diameters of its fibers or changing its Dm, or both (see Figures 2 and 4a). As to the amplitude increase,
the main reason for both LAELS and turbidity data is likely due to fiber mass/length increase.

Of the four data sets of Figure 8, only the one corresponding to the fully formed, aged gel \( t > 1000 \) s has a defined FG concentration (equal to the overall FG monomer concentration, \( c = 0.45 \) mg/mL) and, therefore, can be correctly analyzed by using the global LAELS/turbidity fitting procedure described above. Conversely, for the other three data sets the FG is still polymerizing, and its concentration in the gel is only an unknown fraction of the overall FG concentration. For these samples we followed the method developed in ref 13 where by exploiting the relation between gel concentration and gel parameters \( c = \frac{\pi}{4} \rho d (\frac{D_m}{\omega})^{3-\omega} \) and knowing the parameters \( \xi, D_m, \rho, \) and \( d \) of the aged gel, we showed that it is possible to recover both the effective concentration \( c_{\text{eff}} \) of the polymerizing gel and its parameters. Alternatively, \( c_{\text{eff}} \) could have been estimated by measuring the rate of release of the fibrinopeptides during polymerization,27 but this analysis was not carried out in the present study. We also note that the entire data fitting was carried out by properly taking into account the dispersion factors for \( n \) and \( d n / d c \) (see Supporting Information Appendix A).

The results of these fittings, shown as solid lines in panels (a) and (b) of Figure 8, are rather satisfactory and reproduce accurately the behavior of all the data, as also evidenced by the non systematic residuals reported in panels (c) and (d). A summary of the parameters recovered from this analysis (applied also to data taken at other times not reported in Figure 8) is shown in Table 2. As one can notice, almost all the parameters tend to increase with time, up to saturation levels that occur at \( t \geq 1000 \) s. While this trend is clearly monotone for \( D_m \) and \( c_{\text{eff}} \) at all times, in the case of \( \langle \xi \rangle \) and \( \langle \rho \rangle \) it occurs only at the beginning, whereas for \( \langle d \rangle \) it takes places only during the late phases (thickening phase). Similarly, the fibers mass/length defined as \( \langle M/L \rangle = N_\omega\pi (\frac{\pi}{4}) (\rho)_{\omega} (\langle d \rangle)^2 \) seems to increase only at the later times. Notably, the highest variation takes place for the \( c_{\text{eff}} / c \) parameter, which increases by \( \approx 100\% \) over the reported time range. The overall behavior of the parameters reported in Table 2, which refers to the late phases of the polymerization process after the onset of the network, seems to be consistent with our recent study26 on the early phases of the process well before the formation of the network (see next section). The low density values recovered for the fibers are in line with the original Carr and Hermans findings11 and seem to be also consistent with a recent SANS study where elongated, solvent-filled cavities were proposed to be present in fibrin fibers, although no estimate of their frequency was provided.17 In addition, intrinsic disorder inside the fibers could also contribute to the observed SANS patterns.19

Finally, it should be pointed out that the parameters \( \xi, \rho, \) and \( d \) are reported in Table 2 as average parameters because, in real gels, we always expect to have some polydispersity in \( \xi, d, \) and in \( \rho \) as well (although to our knowledge, there is no data reported in the literature for the latter). As to the pore size, it has already been shown14 that any polydispersity in \( \xi \) shows up in a LAELS experiment as an almost weight-average; therefore \( \xi \approx \langle \xi \rangle \). Similarly, as shown in Supporting Information Appendix C, any polydispersity in \( \rho \) and \( d \) results in a weight-average of the quantity \( \rho d (D_m/d_x) \). If we make the (somewhat arbitrary) assumption that the probability distributions of \( \rho \) and \( d \) are uncorrelated, then we have \( \rho = \langle \rho \rangle , \) and \( d = \langle d \rangle = \langle (D_m/d_x) \rangle ^{1/(3-\omega)} \). Note that when \( D_m \approx 1 \), \( \langle d \rangle \approx \langle (D_m) \rangle ^{1/2} \).

As a final remark, we would like to emphasize that the method so far illustrated relies on the use of a fitting function based on the analysis of the properties of fibrin gels, described as assemblies of cylindrical elements. Thus, the method is prone to be applied well beyond this specific case. To assess its general validity, we have applied it to a number of different types of filamentous networks, generated in silico and again composed of cylindrical units. Namely, we have simulated collagen gels,30 photon band gap networks (PBG),31 and purely in silico networks based on the Voronoi tessellation and the Delaunay triangulation. While a detailed description of the procedures followed for generating these networks can be found in Supporting Information Appendix E, here we report and comment only on the comparison between the various networks parameters recovered with our two-step fitting procedure and the expected ones. These findings are summarized in Table 3, where one can appreciate that except for the parameter \( \xi \), for which there are a couple of notable exceptions, all the fitted parameters are recovered with accuracies better than \( \approx 10-15\% \). Therefore, the method proposed in this work can also be extended to a large variety of biological, synthetic, or other geometrical filamentous networks that can be described as an assembly of cylindrical elements. The only requirement is the knowledge of the network mass fractal dimension \( D_m \) and its pore size \( \xi \).

### CONCLUSIONS

In this work, we have critically examined the Carr—Hermans method11 which has been profitably used for decades for estimating the mass/length ratio \( \mu \), the diameter \( d \), and density \( \rho \) of fibers in filamentous biopolymer networks and in particular for fibrin gels. We have shown that under many circumstances (such as when the fibers are not sufficiently small or long enough, or when polydispersity is present) the applicability of the CH method becomes quite critical, and its results might unfortunately be rather unreliable. In particular, when the network morphology is characterized by a mass fractal dimension \( D_m > 1 \), the errors in the estimates of the various parameters become remarkable (up to several hundred percent), and a different approach must be followed.

For these reasons, we have developed a new method to analyze the turbidity data of fibrin gels, which were taken as a

<table>
<thead>
<tr>
<th>time (s)</th>
<th>( \langle \xi \rangle ) (μm)</th>
<th>( D_m )</th>
<th>( \langle \rho \rangle ) (g/cm³)</th>
<th>( \langle d \rangle ) (nm)</th>
<th>( \langle M/L \rangle ) (Dm/( nm \times 10^3 ))</th>
<th>( c_{\text{eff}} / c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>230</td>
<td>1.27 ± 0.1</td>
<td>1.24 ± 0.01</td>
<td>0.37 ± 0.02</td>
<td>93 ± 5</td>
<td>1.50 ± 0.08</td>
<td>0.48 ± 0.01</td>
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<td>300</td>
<td>1.31 ± 0.1</td>
<td>1.30 ± 0.01</td>
<td>0.42 ± 0.03</td>
<td>85 ± 6</td>
<td>1.45 ± 0.09</td>
<td>0.61 ± 0.01</td>
</tr>
<tr>
<td>430</td>
<td>1.31 ± 0.1</td>
<td>1.33 ± 0.01</td>
<td>0.43 ± 0.03</td>
<td>85 ± 6</td>
<td>1.50 ± 0.09</td>
<td>0.75 ± 0.01</td>
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<td>605</td>
<td>1.30 ± 0.1</td>
<td>1.35 ± 0.01</td>
<td>0.43 ± 0.03</td>
<td>87 ± 6</td>
<td>1.56 ± 0.10</td>
<td>0.86 ± 0.02</td>
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<tr>
<td>730</td>
<td>1.31 ± 0.1</td>
<td>1.36 ± 0.01</td>
<td>0.43 ± 0.03</td>
<td>89 ± 6</td>
<td>1.60 ± 0.10</td>
<td>0.90 ± 0.02</td>
</tr>
<tr>
<td>1480</td>
<td>1.31 ± 0.1</td>
<td>1.37 ± 0.01</td>
<td>0.43 ± 0.03</td>
<td>91 ± 5</td>
<td>1.68 ± 0.09</td>
<td>0.99 ± 0.01</td>
</tr>
<tr>
<td>&gt;3000</td>
<td>1.32 ± 0.1</td>
<td>1.37 ± 0.01</td>
<td>0.44 ± 0.03</td>
<td>90 ± 5</td>
<td>1.70 ± 0.06</td>
<td>1.00 ± 0.01</td>
</tr>
</tbody>
</table>
model system for this study. The method is based on the knowledge of the scattering form factor of the gel and in particular on the knowledge of the gel pore size $\xi$ and mass fractal dimension $D_m$. The latter two parameters can be accurately recovered from low-angle elastic light scattering or estimated by using other techniques, such as for example confocal microscopy or rheometry. The new method works by fitting the turbidity data with a function obtained by numerically integrating the scattering form factor of the gel. In this way the diameter $d$ and density $\rho$ of fibers can be accurately recovered. The method can also be applied with satisfactory reliability even when the values of $\xi$ and $D_m$ are not known with high accuracy. We have shown that almost independently of $\xi$, if $D_m$ is known with a ±0.02 error, the accuracy in the recovery of $\rho$ and $d$ is always within ±10–20%.

When applied to a set of LAELS/turbidity data taken on polymerizing FG solutions, the great potentialities of our method become apparent, with the recovery of the time evolution of the pore size and mass fractal dimension of the gel, and of the density and diameter of its constituents fibers. As shown in Table 2, there is an initial stage ($t \leq 430$ s) during which $\langle \xi \rangle_m$, $D_m$, and $\langle \rho \rangle_m$ increase and reach their final saturation levels, whereas the parameters $\langle d \rangle_s$ and $\langle M/L \rangle$ seems to be almost constant and maybe even decrease at the very beginning (first–second rows). After that, $\langle d \rangle_s$ and $\langle M/L \rangle$ slightly increase and reach their asymptotic values at times $t \sim 100$ s. As to the $c_{all}/c$ parameter, it constantly increases from the very beginning to the end. This overall behavior seems to be only partially consistent with the picture previously reported, where after the formation of the network, the growth kinetics consists of a ripening phase during which the network structure slightly changes and the fibers start to grow, followed by a thickening phase during which the structure remain the same and the fibers become increasingly thicker. Here, it seems that the ripening phase is characterized only by a readjusting of the network structure without a clear evidence of an increase of the fiber diameter or $M/L$. During this phase the increase of the scattering intensity has therefore to be attributed only to an increase of $c_{all}/c$ and $D_m$. We can also try to correlate this behavior with what happens up to the networking time, when fibrin polymerization can be described in terms of a revised model that we have recently proposed. In this model, it is the interconnection of a few threads belonging to hyperbranched "blobs" of thin fibrils that forms the initial permissive network, followed by the collapse of the other branches giving rise to thick (and therefore turbid) but low-density fibers. This transition should therefore correspond to the ripening phase, during which the fibers increase their density but not their diameter, followed by a slower thickness increase at constant density when the remaining FG monomers/polymers still present in solution becomes bound to the established network's fibers. However, although this interpretation is fairly intriguing, further experiments are clearly needed to fully validate this claim.

Finally, although developed for the specific case of the fibrin gels, the method here proposed has a general validity and can be applied to any other filamentous network that can be described in terms of an assembly of cylindrical elements. We have provided several examples of this important feature in Supporting Information, where we have shown that for (simulated) collagen gels, photon band gap networks (PBG), and purely in silico networks based on the Voronoi tessellation and the Delaunay triangulation, the method is able to provide estimates of $\rho$ and $d$ with better than 10–15% accuracy.

### ASSOCIATED CONTENT

#### Supporting Information

Appendix A: dispersion effects affecting the Carr–Hermans method; Appendix B: polydispersity effects affecting the Carr–Hermans method; Appendix C: scattering function for a fibrin gel; Appendix D: error estimates in the fitting of turbidity data; Appendix E: application of the method to other filamentous networks. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.macromol.5b00893.

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Notes
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REFERENCES


(22) De Spirito, M.; Arcovito, G.; Papi, M.; Rocco, M.; Ferri, F. Biophys. J. 2013, 104, 1160–1169.


