



Cite this: *Soft Matter*, 2023, 19, 2780

Modelling network formation in folded protein hydrogels by cluster aggregation kinetics†

Kalila R Cook, ^a David Head ^{*b} and Lorna Dougan ^{*ac}

Globular folded protein-based hydrogels are becoming increasingly attractive due to their specific biological functionality, as well as their responsiveness to stimuli. By modelling folded proteins as colloids, there are rich opportunities to explore network formation mechanisms in protein hydrogels that negate the need for computationally expensive simulations which capture the full complexity of proteins. Here we present a kinetic lattice-based model which simulates the formation of irreversibly chemically crosslinked, folded protein-based hydrogels. We identify the critical point of gel percolation, explore the range of network regimes covering diffusion-limited to reaction-limited cluster aggregation (DLCA and RLCA, respectively) network formation mechanisms and predict the final network structure, fractal dimensions and final gel porosity. We reveal a crossover between DLCA and RLCA mechanisms as a function of protein volume fraction and show how the final network structure is governed by the structure at the percolation point, regardless of the broad variation of non-percolating cluster masses observed across all systems. An analysis of the pore size distribution in the final network structures reveals that, approaching RLCA, gels have larger maximal pores than the DLCA counterparts for both volume fractions studied. This general kinetic model and the analysis tools generate predictions of network structure and concurrent porosity over a broad range of experimentally controllable parameters that are consistent with current expectations and understanding of experimental results.

Received 26th January 2023,
Accepted 17th March 2023

DOI: 10.1039/d3sm00111c

rsc.li/soft-matter-journal

1 Introduction

In the field of colloid and interface science, there is a wide array of systems that are well approximated as colloidal when considering their properties and behaviours. These range from functional colloids in foods which influence dispersibility, stability and structure,¹ the self-assembly of colloidal cube superstructures,² to biological systems such as complex interactions of DNA-lipid complexes in gene delivery^{3–5} or modelling bacteria as colloids to understand their morphology, behaviour⁶ and eventual formation of biofilms.^{7,8} To add to these, folded globular proteins are sometimes also studied within a colloidal framework,^{1,9–14} whereby basic definitions of colloids, they fit the submicron size range (generally sub 10 nm radius, assumed spherical^{15,16}) and can be electrostatically stably dispersed in solution. Globular proteins such as bovine serum albumin (BSA), for example, are often satisfactorily modelled in solution as hard-sphere colloids, with added

potentials to account for complex attractive and repulsive interactions between charged proteins such as electrostatics, van der Waals, hydration and hydrophobic interactions.^{10,11,13,17–23}

Whilst the colloidal approach to proteins is not new, its application in the context of the growing field of folded protein-based hydrogels is less explored. Indeed, proteins have long been utilised as the constituent building block for hydrogels,^{24,25} and in common with other systems, protein-based systems employ two main types of crosslinking strategies: chemical (irreversible or permanent) and physical (reversible).²⁶ However, gelation is more often initiated by physical methods of denaturation of the protein *e.g.* by changing the pH,²⁷ temperature^{27–29} or electrostatics²⁰ of the system, or rather gelation progresses by chemical- or force-induced unfolding of protein domains.^{30–33} While both partial and full denaturation-induced protein hydrogel formation can provide a range of mechanical and structural properties for hydrogels, this approach can lead to a loss of native globular structure and subsequently a loss of the inherent protein biological function.^{20,34,35} In terms of modelling denaturation-induced protein hydrogel formation, the potential for treating the systems colloiddally may be lost, since new factors can dominate the system kinetics such as increased hydrodynamic radius and/or change of conformation to a non-globular state, complex resultant excluded volume effects³⁶ and change in inter- and intramolecular interactions, perhaps leading

^a School of Physics and Astronomy, Faculty of Engineering and Physical Sciences, University of Leeds, Leeds, UK. E-mail: L.Dougan@leeds.ac.uk

^b School of Computing, University of Leeds, Leeds, UK

^c Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, UK

† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d3sm00111c>



to supramolecular aggregation and differences to the bulk properties of the systems.³⁷ It is therefore recognised that there are limitations to taking a colloidal view of these systems. However, as covered comprehensively in the recent review article by Stradner *et al.*,¹³ in the right circumstances, where (quasi)-spherical globular proteins retain their tertiary conformation,^{38,39} this approach is appropriate and can bring valuable insight into the mechanisms leading to observed structures and mechanics of protein hydrogels across the lengthscales. Indeed, a growing field has emerged which exploits folded globular proteins for the creation of hydrogels, using irreversible crosslinking to control network formation.^{40,41} For example, the maximum coordination of folded proteins in gels (defined as the maximum allowed number of chemical crosslinks per domain) can be controlled by tuning the number of site-specific binding residues on the surface of the proteins.⁴² Such systems lend themselves well to a colloid-based modelling approach, and there is now a growing body of literature on the assembly, rheology and structure of such hydrogels.^{31,43–45}

The benefit of adopting a coarse-grained representation of proteins is that it allows for network formation mechanisms to be examined over the full course of gelation, reducing computationally expensive simulations of complex features of proteins, such as their secondary structure and so called ‘mechanical clamp motifs’ which confer their mechanical robustness,⁴⁶ that may be less of interest at larger lengthscales.¹³ Furthermore, regarding proteins as colloids grants the use of established models of cluster aggregation,^{47–52} allowing for efforts to be focused on achieving longer simulation timescales to understand how the networks evolve and how this impacts the final network structure. Indeed, there are a limited number of works that have used this concept to model globular protein hydrogels in this way,^{42,53,54} where keeping the protein monomer unchanged during gelation enables other important aspects of network formation to be deconvoluted, such as the effect of different initial size or shape of the monomer, extent of crosslinking and volume fraction. However, it seems thus far, there remains a lack of understanding of the dominant network formation mechanisms on the full gelation profile of these gels. Recent work by Hanson and Dougan delves into the structural characteristics obtained by simulating the formation of folded globular protein-based networks with variable parameters, such as crosslink length and number, while keeping the shape of the protein building block unchanged.⁴² As the binding sites are located explicitly at the surface of each protein, their local geometric organisation on the surface of the protein is a parameter of importance. This work highlights the significant control that can be achieved on the network architecture without needing to substantially change the protein building block (as is now more often reported^{30,31,39,43}), including by controlling the mechanical stability of the protein,^{32,39,55} the aspect ratio of the building block,⁵⁶ colloidal linker regions (*e.g.* length, flexibility and concentration)^{42,57,58} and the crosslinking ability of the protein.⁵⁹ However, there are still limits to the reaction timescales that can be explored simply by the computationally intensive nature of including complex dynamic potentials and interactions in these molecular dynamic simulations.

Here we present a kinetic lattice-based model which models folded globular proteins as colloids and simulates their irreversible chemical crosslinking into gels. By exploiting the parallels between well-established colloidal models and globular protein solutions, we have been able to strip back non-essential complexities in the gelation model by a highly coarse-grained approach, whilst qualitatively maintaining the network formation region of the gelation profiles that we observe in our real experimental systems.^{32,39,59} Our model is thus intermediate between atomistic simulations and continuum-level finite element descriptions.⁶⁰ From the refined simplicity of our model, we are able to hone in on the effect of changing experimentally available variables of reaction probability (affiliated with reaction rate) and protein volume fraction on the development of permanent network architecture up until complete system crosslinking, even approaching the reaction-limited regime where computational timescales are at their longest.

From this model, we are able to identify the critical point of gel percolation and use this to construct a fitting for predicting the final network structure. By our bespoke equation for fractal fitting and measurements of final gel porosity, we show that our predictions on the network structure in the final state are accurate. Additionally, we demonstrate how the network structure at the percolation point governs the final network architecture,⁶¹ further showing the predictive power of our model from early on in the gelation process.

2 Methods

2.1 Modelling cluster–cluster aggregation

2.1.1 The cluster–cluster aggregation model. We have designed a simple 3D kinetic model for colloidal monomer aggregation into gels, where each cubic monomer represents a folded globular protein. The key processes to consider are the random diffusion of monomers, their collisions and potential crosslinking (aggregation) into clusters and the subsequent, iterative diffusion and aggregation of these clusters. In order to achieve this, we considered discrete-time stochastic processes, allowing for complete system gelation within short computational timescales. Here, we define gelation as the point at which all monomers and clusters of monomers are crosslinked together into one final cluster.

The initial parameters are N , the number of monomers in a cubic, lattice-based simulation box of size L^3 with periodic boundary conditions (PBCs) and cells in discrete units of monomer diameters. The monomer volume fraction of the simulation box, ϕ , is set initially by choosing N for a given L , where $\phi = N/L^3$. Since L is fixed at 64 for all simulations, N must differ to give the desired value of ϕ (*e.g.* a 4% monomer volume fraction requires $N = 10\,486$ for L fixed at 64). We can confirm that this chosen L is not subject to finite size effects, and this data can be found in Fig. S3 in ESI.† R is chosen as the probability with which adjacent monomers crosslink, ranging from 100% (diffusion-limited cluster aggregation (DLCA)) to 0.2% (approaching reaction-limited cluster aggregation (RLCA)).



Initialisation of the system proceeds by trialing random 3D position coordinates for the N monomers. So long as monomers do not overlap in space, those monomer positions are 'allowed'. Where any monomer overlaps with another in space, that position is not allowed, and the position-assigning loop is trialed again. This loop is repeated until all N monomers have been situated in the simulation box. At this point, a list of clusters is initialised with N clusters, each of size one, where size is measured in number of monomers that form a given cluster.

2.1.2 Monomer diffusion and cluster aggregation. After initialisation, the simulation begins with an attempt to move all monomers (or clusters of size one monomer) by one unit cell in the first time step. The probability with which a randomly selected cluster, i , made up of M_i monomers, is successfully moved in the time step is given as^{62,63}

$$P_i = M_i^{-\alpha},$$

where $\alpha = 1/d_f$ and d_f is the fractal dimension of the final gel with an initial guess of 2.4. This has the overall effect of slowing the motion of larger clusters over the course of the simulation (noting also that the probability is directly proportional to the translational diffusion coefficient of the cluster, $D(M_i)^{62,63}$). If a cluster can be successfully moved based on this probability (very likely in the initial case of monomers), its direction of motion is randomly assigned and can be in one of six directions in the 3D cubic lattice. If the cluster can then be moved without overlapping onto another, it will finally be moved one lattice space.

Once an attempt has been made to move every cluster in that time step, the program checks whether any clusters are adjacent, *i.e.* whether they share a side in the 3D system lattice. Any clusters that are adjacent will aggregate (merge) with a 'reaction probability', R , as set initially by the user. The simulation time step then increases by one, and the cluster diffusion and aggregation process is repeated until the final time step is reached, where all monomers are incorporated into the percolating cluster.

2.2 Outputs

2.2.1 Percolation point. Percolation (or gelation) can be defined as the point at which a system-spanning cluster emerges, marking a sol-gel transition in colloidal systems.^{64–68} Since this model has periodic boundary conditions, the percolation point here is specifically defined as when a cluster is first large enough that it traverses the size of the system box in all three dimensions. Clusters are monitored for percolation at every time step. Assuming only one cluster can percolate at a time, the size of the percolating cluster at the critical percolation time is captured and its growth monitored until it reaches maximal size (fully 'crosslinked') by the end of the simulation. Example simulation snapshots of this course of gelation are presented in Fig. 1a. There may be a lag time at the beginning of the simulation where the percolating cluster has no size since it has not formed yet. This elapsed time is annotated as the 'percolation time, τ ' on the example simulated gel curve of Fig. 1b, starting at (1) time zero

and ending at (2) the critical percolation point, denoting the emergence of a non-zero-sized percolation cluster. By (3), the system is fully crosslinked. This simulated gel curve is also presented on a log time axis in ESI,[†] Fig. S1.

By changing the initial conditions variables of ϕ and R , the progression of gelation and the final gel network structure can be altered, as shown in Fig. 2a. Fig. 2b shows how the percolation time and subsequent time for growth of the percolation cluster to a fully crosslinked network are extended as ϕ is decreased for each system but R is fixed at 100%. Fig. 2c shows how the percolation time and further growth are extended when ϕ is instead fixed at 4%, but R is decreased from 100 to 0.2%. Fig. 2b and c are also presented on log time axes in ESI,[†] Fig. S1.

2.2.2 Fractal dimensions from box counting. One way to define the fractal dimension of the system as a whole is using the Hausdorff–Besicovitch, or box-covering, dimension.^{69,70} To obtain this dimension at any point in the simulation, we have written and implemented an additional algorithm to pave the 3D system with measuring cubes of unit size, r . Out of the total cubes in the system, those that contain at least any part of a cluster or a monomer are counted as 'occupied', and this occupancy number, $O(r)$, is recorded. Classically, a 'geometric step' box-covering algorithm^{71,72} is used that begins with the largest 3D box size of $r = \text{system size}$ (cubic number in units of number of boxes), records the associated $O(r)$ and then iterates this loop by repeatedly halving the size of r until the minimum box size is reached ($r = 1$). Thus any invariance in the cluster structure across the lengthscales of r , otherwise known as fractality, can be captured and quantified. In the original box-covering method, the Hausdorff–Besicovitch fractal dimension, d_f , can then be obtained simply as the straight-line gradient of the logs of these measured quantities as such,

$$d_f = \frac{\log(O(r))}{\log(r)}.$$

Halving the box-covering box size in this method leads to very few data points available for fitting at low r for small system sizes. Low r data provide the fine-scale fractal dimension of the network structure ($d_f < 3$), whereas it would be expected that high r data would contribute more to a higher fractal dimension where the lengthscale becomes so coarse that the final cluster appears as a homogeneous solid ($d_f \rightarrow 3$).⁷³ Therefore, in order to reliably capture the fine-scale fractal dimension and the crossover between lengthscales, box-counting here was instead adapted to capture all box sizes between $r = 1$ and the system size, providing a full set of data points for any system size. Since some of these box sizes lead to partial boxes near the system boundaries, these partial boxes are fractionally weighted according to the method of So, So and Jin.⁷¹

Having a uniform sampling of data points over the full available range of r highlights a curve in the data and the need for a more advanced fitting function than the straight-line extraction of the Hausdorff–Besicovitch fractal dimension. Therefore, we have constructed the following equation that



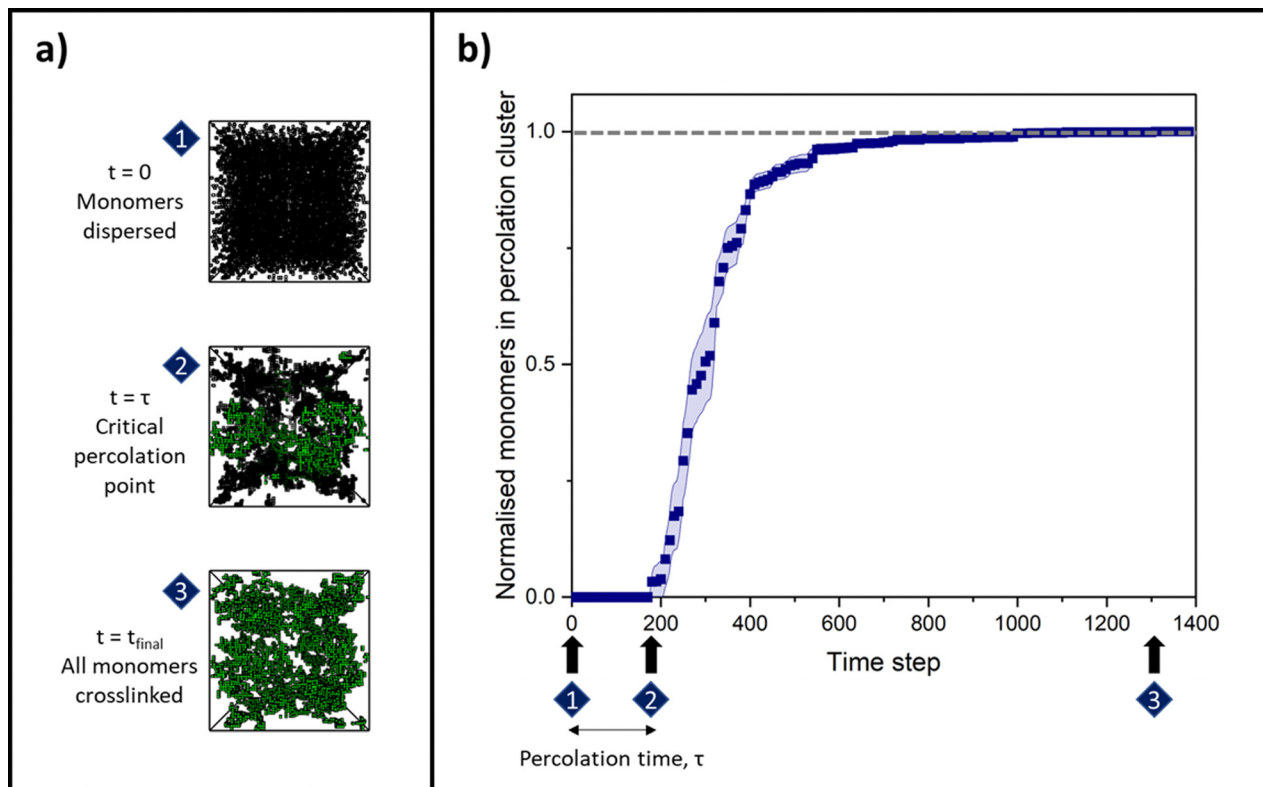


Fig. 1 (a) Simulation snapshots of a 4% volume fraction system with a monomer–monomer reaction probability of 100%, with the largest cluster at that point in time presented in green and non-percolating intermediate clusters and monomers presented as black nets. (1) The initial state of randomly distributed monomers; (2) the critical percolation point at which a cluster has grown large enough that it first spans the three dimensions of the simulation box; (3) the final, fully crosslinked gel state, determined when the normalised number of monomers in the percolation cluster equals 1.0 (grey horizontal dashed line). (b) An example raw data curve of change in proportion of monomers in the percolating cluster over time, averaged over 10 simulation runs of a 4% volume fraction system with a monomer–monomer reaction probability of 100%. The time steps at which the simulation snapshots occur from (a) are denoted, where the elapsed time between (1) and (2), before a non-zero sized percolation cluster first forms, is called the ‘percolation time, τ ’.

better fits the data, from which the fine-scale fractal dimension, d_f , can be obtained as a fitting parameter:

$$O(r) = \frac{\left(\frac{L}{r'}\right)^3 + \left(\frac{L}{r'}\right)^{d_f}}{\left(\frac{r}{r'}\right)^{\frac{3}{\alpha}} + \left(\frac{r}{r'}\right)^{\frac{d_f}{\alpha}}}, \quad (1)$$

where L is the length of the 3D system size, r' is the lengthscale in r at which there is a transition to the fine scale and fractal characteristics emerge and α is the non-zero cross-over width of this transition, capturing curvature in the data. Plotting logs of $O(r)$ against r as before and fitting with the log form of eqn (1) allows for d_f to be extracted. This two-regime fitting form robustly interpolates between fractal and non-fractal limits, and examples of resultant fitted box-covering plots for different volume fraction and reaction probability conditions are given in ESI,† Fig. S4.

2.2.3 Pore size distribution. The configuration of voids within the network can be analysed by computational algorithms employing varying degrees of sophistication depending on the level of characterisation required, potentially including *e.g.* the connectivity between large void spaces or ‘pores,’ and the diameters of ‘throats’ between them.⁷⁴ A more straightforward first

analysis, sufficient for our purposes, is a basic measure of the distribution of pore sizes, calculated as follows. For a given network configuration, L^3 spherical pores are placed with centres on a cubic $L \times L \times L$ lattice that coincides with the simulation grid. Each pore diameter is then increased from zero until the pore first touches any monomer in the network. Pores with diameters less than the monomer diameter are removed at this stage. All remaining pores are sorted in order of decreasing diameter and overlapping pores are removed, starting from the smallest, and with the smallest of overlapping pairs removed. This generates a unique distribution of the largest non-overlapping pores, with varying diameters that can be gathered into the pore size distribution. The simplicity of this method permits rapid evaluation of a measure of pore size distribution without requiring significant implementation or simulation time, and produces a representative estimate of large pores within the network.

3 Results and discussion

3.1 Network percolation

Colloidal aggregation simulations were run to completion (fully crosslinked) at monomer volume fractions of 3, 4, 5, 6 and 8%



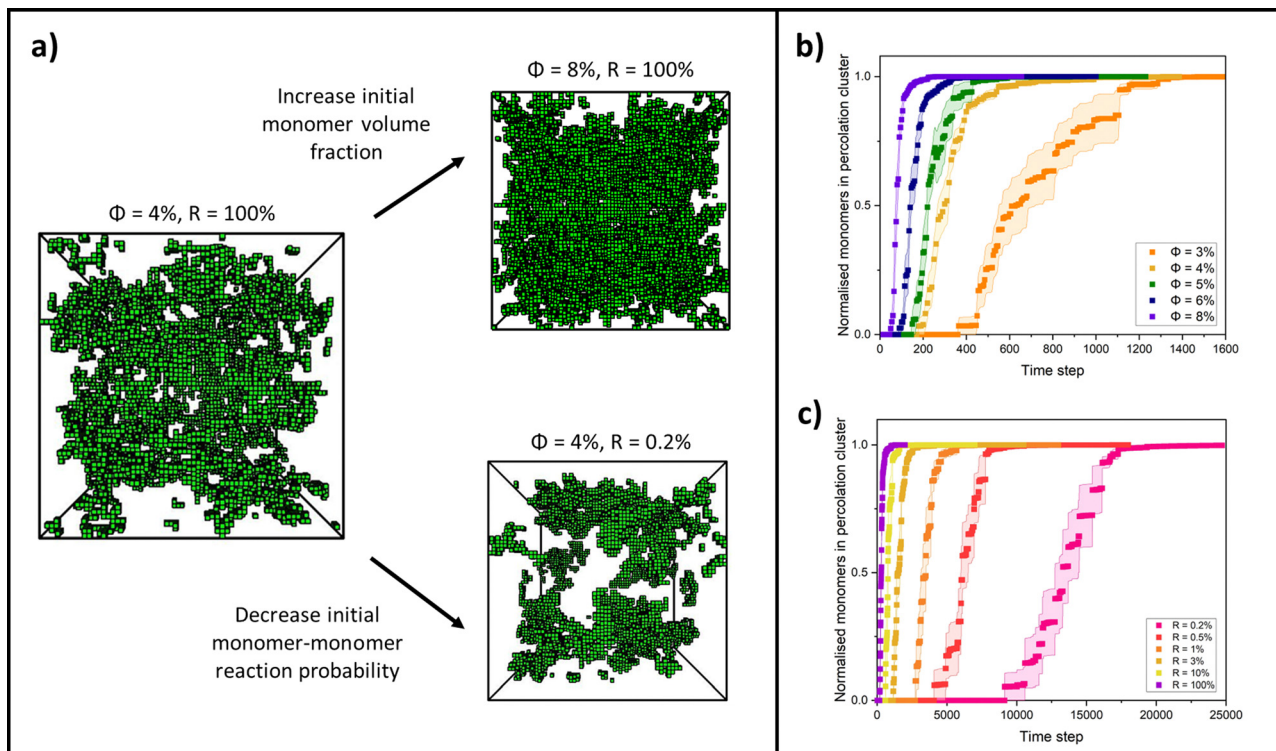


Fig. 2 (a) Simulation snapshots of final, fully crosslinked gels formed from different initial conditions, where ϕ is the monomer volume fraction and R is the monomer–monomer reaction probability. (b) Change in proportion of monomers in the percolating cluster over time for monomer volume fractions ranging from 3 to 8%, all for a monomer–monomer reaction probability of 100%. (c) Change in proportion of monomers in the percolating cluster over time for 4% monomer volume fraction systems ranging from 0.2 to 100% monomer–monomer reaction probabilities. Curves for reaction probabilities between 15 and 100% are not plotted for clarity since they begin to overlap.

(representative of experimental folded protein-based hydrogel volume fractions^{32,39,59}) and each at a wide range of monomer–monomer reaction probabilities between 0.2 and 100%. From these simulations, the time at the precise point of percolation (as by our definition), τ , was captured and plotted against reaction probability, R , for each monomer volume fraction, ϕ . Example snapshots of some of these simulations at the percolation point are presented in Fig. 3a with the percolating cluster shown in green and the remaining non-percolating monomers and clusters presented as black ‘wireframes’. Fig. 3b shows the percolation time data on a natural log plot, where each data point represents an average of ten simulation runs of a gelling system with particular initial parameters of ϕ and R . The first trend to note is that the onset of percolation happens sooner with increasing volume fraction across all reaction probabilities (the data sets shift downwards with increasing volume fraction). This is as predicted, since a higher volume fraction of monomers should lead to a higher frequency of monomer–monomer interactions, leading to faster percolation of the network.

The data sets are fitted with a reciprocal function in its log form,

$$\ln(\tau) = \ln(A + BR) - \ln(R), \quad (2)$$

where A and B are constants that relate to the reaction and diffusion limited regimes, respectively. Using this fitting function,

the limiting reaction cases can be realised. As $R \rightarrow 1$ (100%), eqn (2) becomes $\ln(\tau) \sim \ln(A + B)$, so τ is constant and no longer depends on R , as is expected in the DLCA regime. On the other hand, as $R \rightarrow 0$ (0%), eqn (2) becomes $\ln(\tau) \sim \ln(A) - \ln(R)$, which, comparing to the equation of a straight line, gives an inverse dependence of τ on R , as is expected in the RLCA regime (*i.e.* the network formation time is entirely controlled by the reaction probability).

This form of reciprocal fitting, with an inverse unitary power law scaling between τ and R , follows that observed in analogous experimental folded protein gel systems, where photochemical crosslinking of bovine serum albumin (BSA) protein units for a volume fraction of 7.5% yielded a power law fit of gel time with reaction rate of -1.08 ± 0.21 .⁵⁹ Our fitting builds on this experimental fitting with the inclusion of the B constant term which accurately models the high R portion of the data sets, up to 100% reaction probability – beyond that obtained experimentally. Additionally, by taking the criterion that $\frac{A}{R} > B$ from eqn (2), we can obtain a crossover between cluster aggregation mechanisms as $R_{CO} = \frac{A}{B}$. This crossover region is marked for each volume fraction in Fig. 3b. The dashed black line through these points is drawn as a guide to the eye, separating the graph into a RLCA-driven regime to the left of it and DLCA to the right.



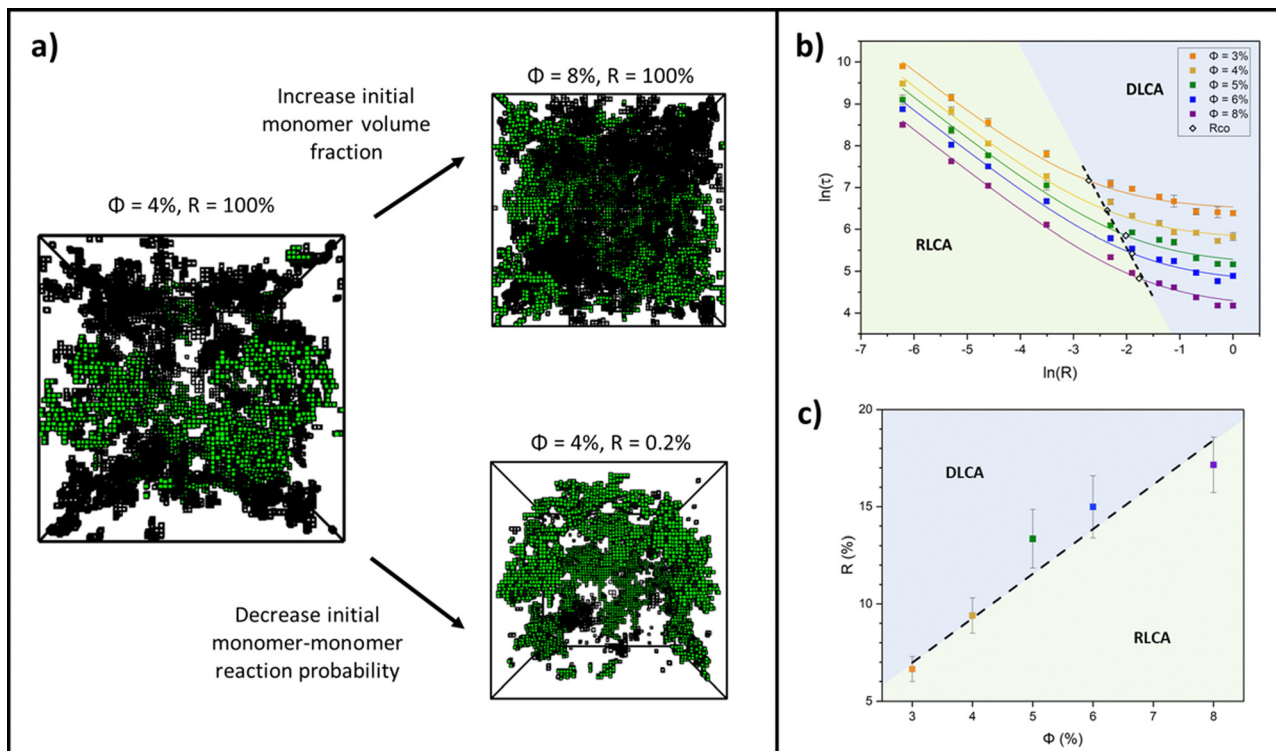


Fig. 3 (a) Simulation snapshots of gelling systems at the percolation point formed from different initial conditions, where ϕ is the monomer volume fraction and R is the monomer–monomer reaction probability. The percolation cluster presented in green and non-percolating clusters/monomers presented as black ‘wireframes’. (b) Natural log plot of percolation time, τ , versus monomer–monomer reaction probability, R , between 0.2 and 100% for ϕ between 3 and 8%. Each point is an average of ten simulation runs. The crossover between reaction regimes, R_{CO} , for each ϕ is denoted by open diamond symbols. The dashed black line is a guide to the eye as a fitting through these points. (c) The reaction regime crossover points taken from (b) plotted as a function of R versus ϕ and fitted with a straight line. In the log form of this graph, the straight line fit yields a unitary power law of 1.0 ± 0.1 .

Fig. 3c more clearly shows the reaction probabilities at which the crossover between cluster aggregation regimes is found by fitting eqn (2) for each volume fraction. In its log form, for this range of volume fractions, we can fit a straight line with a unitary power law of 1.0 ± 0.1 . Here we see that lower volume fractions of monomers exhibit faster turn off from reaction- to diffusion-limited cluster aggregation *i.e.* the transition happens at lower reaction probabilities. This trend is to be expected, since at lower volume fractions, monomers are required to diffuse further before meeting another monomer and potentially aggregating, pushing the system towards the DLCA regime. Mirroring Fig. 3b, we can again see that the graph can be separated into regions, where above the dashed trend line, one would expect gelation mechanisms to happen in the DLCA regime and below it in the RLCA regime.

As an additional point of interest, another useful quantity for understanding the network structure at the percolation point is the cluster size distribution. We observe that this depends on the reaction probability and present an exemplar cumulative frequency plot of the cluster size distribution for a volume fraction of 6% and reaction probabilities of 100 and 0.2% in ESI,† Fig. S2. We find that a power law can be fitted to the mid-range cluster sizes of the $R = 100\%$ data, though further work would be required to systematically quantify changes to the cluster size distribution and its scaling.

To more closely examine the effect of monomer volume fraction on the percolation time, we next plotted this for each reaction probability in Fig. 4a. For this range of volume fractions, each reaction probability clearly exhibits a strong inverse linear trend in the percolation times with volume fraction. This is to be expected, with higher volume fractions of monomers increasing the frequency of interactions by proximity, leading to faster percolation of a network across the system box. We note also that as the reaction probability is increased, the plots shift towards faster percolation times, as expected, but also begin to converge, almost overlapping onto each other. This observation is indicative of reaction probabilities that are sufficiently high that the gelling systems are pushed into the DLCA regime – the percolation times have little dependence on the monomer–monomer reaction probability.^{49,75,76}

From these results, we take the gradients for each reaction probability to analyse the rate of change in percolation times with monomer volume fraction in Fig. 4b. Interestingly, we see that this rate of decrease in percolation time with volume fraction increases with reaction probabilities (the gradients get steeper). This relationship itself fits an inverse power law, with an extracted exponent of 0.078 ± 0.004 . We currently have no theoretical basis for this exponent and highlight this observation as a potential goal for future theory to derive and explain.



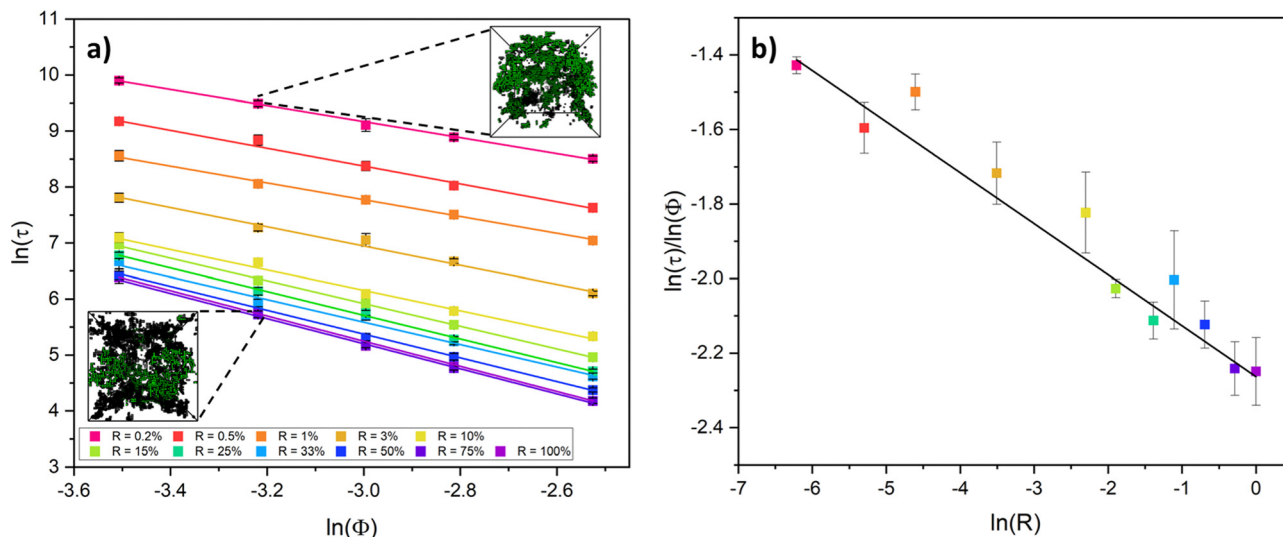


Fig. 4 (a) A natural log plot of percolation time, τ , versus monomer volume fraction, ϕ , between 3 and 8% for a range of monomer–monomer reaction probabilities, R , between 0.2 and 100%. Each point is an average of ten simulation runs. The inset simulation snapshots next to their respective data points ($\phi = 4\%$, $R = 0.2\%$ top and $\phi = 4\%$, $R = 100\%$ bottom) show how the network structure looks quite different at the percolation point for extreme reaction probabilities. (b) The absolute values for the extracted straight-line gradients from (a) are plotted against $\ln(R)$, revealing a weak inverse power law relationship.

3.2 Fractal dimensions

For each aggregation simulation run in Section 3.1, box-covering data of r versus $O(r)$ was collected at the following three system structures and time points: the percolation cluster at the percolation point; the full system (percolation cluster and intermediate clusters) at the percolation point; and the whole system at the end, fully crosslinked state. From these data, extracted values for d_f were then plotted against monomer volume fraction for extreme values of R , 0.2% and 100%, to quantify how the network structure differs in the RLCA and DLCA regimes, respectively. Additionally, d_f was plotted for $R = 33\%$ to see if, as Fig. 3b (and c) predicts, the system has entered the DLCA regime for all volume fractions and therefore if the network structure starts to correlate with that at $R = 100\%$. Each graph in Fig. 5 presents all these reaction probability results for (a) the percolation cluster, (b) the whole system at the percolation point and (c) the whole system on the final fully crosslinked states, alongside example simulation snapshots of the network structure for a system with $\phi = 3\%$ and $R = 33\%$.

Looking at values for d_f on the whole system at the percolation point and at the end state in Fig. 5b and c, there is clearly a decrease with volume fraction in all cases. In previous studies, it is often suggested that, as very high monomer volume fractions are reached, the system becomes more densely packed, and so it is expected that the fractal dimension of the system would increase towards three.^{77–80} We believe that our opposite observation is due to our two-regime fit and fractal dimension extraction method of the box-covering data. As the volume fraction is increased, we observe that the crossover from the fine-scale fractal region of the data occurs at smaller lengthscales of r (see ESI†). As a result, the large- r regime,

where $O(r)$ scales as r^{-3} , increasingly moves to lower r as ϕ increases, eventually covering the full range of r . Therefore the expected dimension of three is recovered for high volume fractions, not by a smooth increase in d_f , but rather by the crossover length moving to zero r and the fractal range being eliminated from the data.

We consider the varied state of the systems at the percolation point in Fig. 5b and c. At the beginning of all simulations, the system begins with the smallest objects being dispersed point particles (monomers) with fractal dimensions of zero. As the monomers aggregate into clusters over time, the fractal dimension of the system increases from zero. For systems at the higher monomer volume fractions, the critical percolation point is reached faster, and thus the measured d_f at this point will be lower. Further to this, we note that there is a sizeable contribution from smaller, non-percolating clusters at the percolation point which will also act to lower the overall fractal dimension of the whole system. For the lower monomer volume fractions, the system takes longer to percolate, by which point there are fewer of these non-percolating clusters, and thus the fractal dimension of the system is higher than that at the same monomer–monomer reaction probability but higher volume fractions. We also recognise our absolute values of d_f differ from those in the literature for similar models that were calculated without counting partial boxes as here, and fitted to using a different functional form. However, our discussion is based on observed trends rather than absolute values, and we expect these trends to hold independent of the measurement protocol.

Considering fractal dimensions for just the percolation cluster in Fig. 5a, there is no clear trend with monomer volume fraction for any reaction probability. However, it may be noted



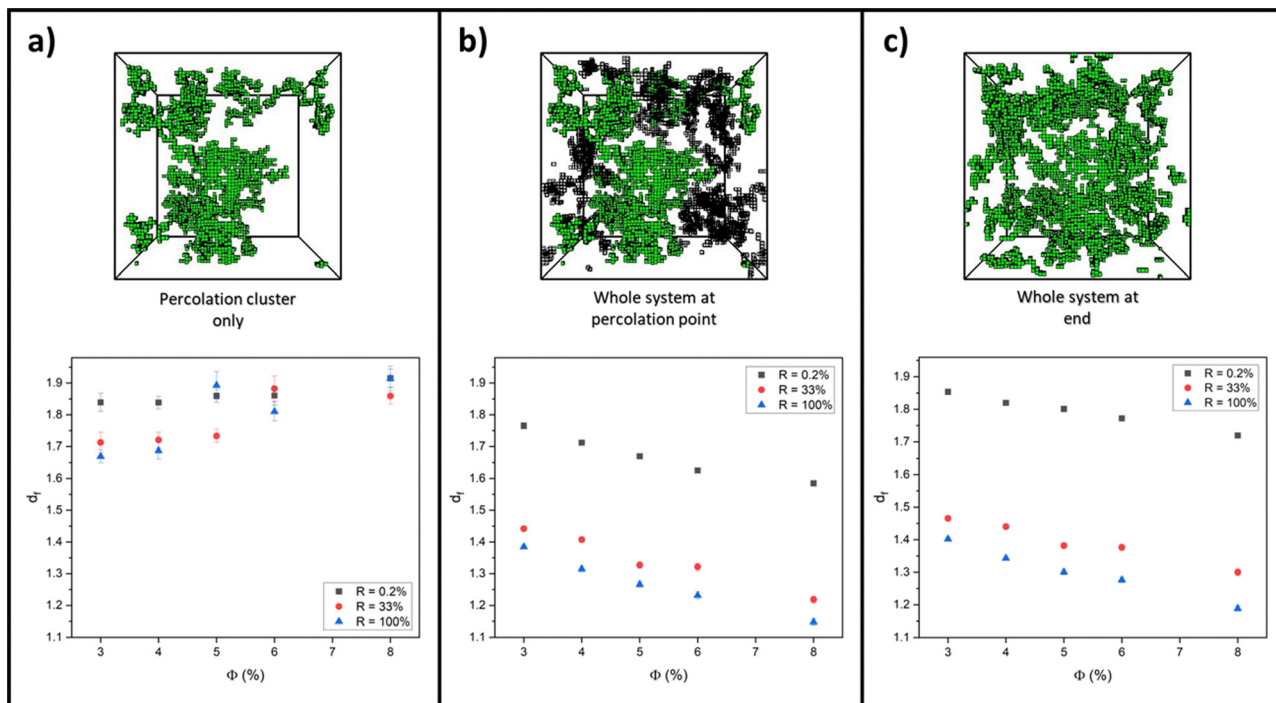


Fig. 5 Graphs of fractal dimensions, d_f , averaged over ten simulation runs at volume fractions, ϕ , between 3 and 8% at reaction probabilities, $R = 0.2\%$ (grey), 33% (red) and 100% (blue). $R = 0.2\%$ is representative of an RLCA system, while $R = 33\%$ and 100% are representative of DLCA systems. Each plot presents these values at (a) the percolation cluster at the percolation point, (b) the whole system at the percolation point and (c) the whole system at the end, fully crosslinked state. Example simulation snapshots for a system with initial parameters of $\phi = 3\%$ and $R = 33\%$ are presented above each graph to show how the systems can look different at these different time points and system structures.

that overall, the values for d_f are higher than those observed for the whole system plots of Fig. 5b and c. If indeed, small clusters are responsible for decreasing the overall fractal dimensions observed when the whole system is considered, this may explain this observation.

Comparing values for d_f on the whole system at the percolation point and at the end state in Fig. 5b and c, it is clear to see that the RLCA regime of $R = 0.2\%$ yields higher fractal dimensions for all volume fractions, as would be expected for the dense clustering in RLCA.^{49,76,81,82} Quantifying this on the end states, we see that at 3% volume fraction, this increase in fractal dimension from $R = 100\%$ to $R = 0.2\%$ is measured as 0.45 ± 0.01 , and this increase is marginally augmented as the volume fractions increase up to 0.53 ± 0.02 at 8% volume fraction. Further to this, we recognise the closeness in values for d_f between $R = 33\%$ and 100% in support of our prediction that $R = 33\%$ systems enter the DLCA regime and thus show similar structural characteristics to $R = 100\%$ systems.

Lastly, we recognise how similar the fractal dimensions are for all volume fractions and reaction probabilities when looking at the whole system at the percolation point and on the fully crosslinked state in Fig. 5b and c. Albeit the values being slightly lower at the percolation point, due to the contribution of non-percolating clusters, this trend indicates how the network structure at the percolation point largely governs the final network structure.^{83,84} This is regardless of the widely varying non-percolating cluster mass that is yet to add to the

percolation cluster and is dependent on the monomer reaction probability and volume fraction.

3.3 Pore sizes in final network structure

Building on the results thus far, Fig. 6 shows examples of largest pore distributions of (a) final-state 4% and (c) 8% volume fraction systems for an RLCA and DLCA system at $R = 0.2\%$ (left snapshots) and $R = 33\%$ (right snapshots), respectively. It can be seen immediately in (a) that the 0.2% RLCA gels have larger maximal pores than the DLCA counterparts for both volume fractions because of the dense clustering and corresponding vast inter-cluster spaces. Fig. 6b and d presents histograms of the largest pore size distributions for the 4% and 8% volume fraction systems, respectively. These clearly show how the DLCA regime reaction probability of 33% yields a distribution of largest pores at smaller radii than at RLCA at $R = 0.2\%$ where the distribution widens and shifts to larger radii. We note also that pore distributions for $R = 100\%$ are very similar to those at $R = 33\%$ for both volume fractions (refer to Fig. S1, ESI[†]), as we anticipated from the evidence thus far that monomer–monomer reaction probabilities between these values should produce gel structures in the DLCA regime. Moreover, it is clear that as the volume fraction is increased between Fig. 6b and d, the largest pore size distributions are shifted to smaller radii for both reaction probabilities, though the cumulative count of these distributions are increased around ten-fold. This is a logical observation, since increased



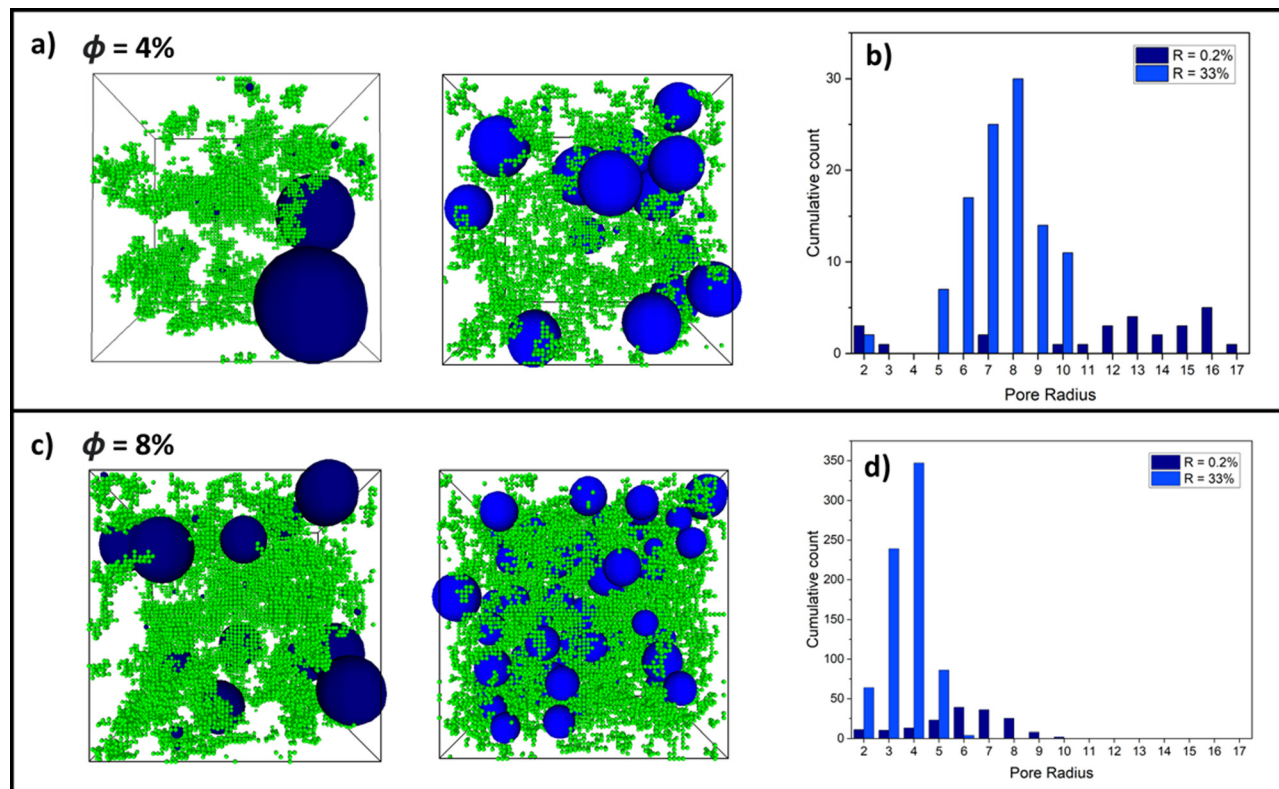


Fig. 6 Snapshots of some fully crosslinked states for (a) 4% and (c) 8% volume fraction (ϕ) gels with monomer–monomer reaction probabilities, R , of 0.2% (left) and 33% (right). Protein monomers are represented as green cubes and pores are represented in blue. Alongside these are the respective histograms of cumulative count of pore size distributions for 10 final states of crosslinked gels at volume fractions of (b) 4% and (d) 8%.

monomer volume fractions would reduce the remaining inter-cluster space.

4 Conclusions

Colloidal aggregation simulations were completed for a model of folded protein hydrogels at monomer volume fractions, ϕ , of 3, 4, 5, 6 and 8% and each at a wide range of monomer–monomer reaction probabilities, R , between 0.2 and 100%. From these simulations, we identify the critical point of gel percolation, explore the range of network regimes covering diffusion-limited to reaction-limited cluster aggregation network formation mechanisms and predict the final network structure, fractal dimensions and final gel porosity. The percolation time, τ , was captured for each R and ϕ . We reveal two distinct regions which are described by DLCA and RLCA and a crossover region between these cluster aggregation mechanisms as a function of ϕ . For each R , a clear inverse relation is observed in the percolation times with ϕ . We explore the structure of the protein networks in more detail by examining the fractal dimensions of the percolation cluster, the whole system at the percolation point and the whole system on the final fully crosslinked states and show how the final network structure is governed by the structure at the percolation point, regardless of the broad variation of non-percolating cluster

masses observed across all systems. An analysis of the pore size distribution in the final network structures provides details of largest pore distributions at different reaction probabilities representative of RLCA and DLCA systems, revealing the RLCA gels have larger maximal pores than the DLCA counterparts for both volume fractions studied.

This approach is important because it allows us to explore the wide design space of network formation in protein hydrogels. Such an approach, to explore reaction- and diffusion-limited cluster aggregation, has proved successful in other biological systems, including modelling bacteria to understand their morphology and behaviour and DNA–lipid complexes in gene delivery. This study on folded protein hydrogels sets the stage for future studies to exploit cluster aggregation to create more complex network architectures, including patterned systems,^{85–87} which might be explored in applications.

Whilst there is already some understanding of the effect of reaction rate and colloidal volume fraction on porosity of gels,^{88–91} what we present here is a general kinetic model and analysis tools which allow for accurate prediction of network structure and concurrent porosity from the initial selection of system parameters. Gel structures which yield percolating pore networks (*e.g.* low monomer volume fractions)⁹⁰ are important for applications where diffusion through the gel is important, such as in drug delivery and nutrient accessibility for in cell-seeded gels.^{40,92–98} Pore size and distribution are important



considerations in tissue engineering, for example where certain cell types need pores of particular sizes for effective growth and tissue regeneration.^{99,100} Being able to control and reproduce the properties of porous gel structure is important for regulating seeded cell properties and behaviour, including proliferation, migration, differentiation and phenotypic variation.^{92,93,101} The ability of our gelation model analyses to predict network structure and pore size distributions from the outset has apparent potential in designing and tuning gel properties for such applications. Furthermore, with additional parameterisation, we expect this model can be extended to describe a broader range of protein-based hydrogels.

Author contributions

All authors conceived the work. K. C. developed and completed the modelling with support from L. D. and D. H. and K. C. led writing of the paper with support from D. H. and L. D.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

K. C. is supported by an Engineering and Physical Sciences Research Council (EPSRC) PhD studentship through the Soft Matter and Functional Interfaces Centre for Doctoral Training (SOFI CDT) (EP/L015536/1) and L. D. through an EPSRC fellowship (EP/P02288X/1) and ERC Consolidator Fellowship with UKRI guarantee (EP/X023524/1). This work was undertaken on ARC3, part of the High Performance Computing facilities at the University of Leeds, UK. The data set for Modelling Network Formation in Folded Protein Hydrogels by Cluster Aggregation Kinetics can be found here <https://doi.org/10.5518/1323>. We are grateful to Dr Ben Hanson for his useful discussions and considerable input in constructing the equation for finding fractal dimensions. Thanks to members of the Dougan group for helpful discussions, in particular Dr Matt Hughes. Thanks also to Dr Holly Linford for colour-editing the gel pore snapshots.

Notes and references

- 1 A. Ashfaq, K. Jahan, R. U. Islam and K. Younis, *LWT*, 2022, **154**, 112667.
- 2 C. L. Kennedy, D. Sayasilpi, P. Schall and J. M. Meijer, *J. Phys.: Condens. Matter*, 2022, **34**, 214005.
- 3 R. Podgornik, H. H. Strey and V. A. Parsegian, *Curr. Opin. Colloid Interface Sci.*, 1998, **3**, 534–539.
- 4 H. Liang, D. Harries and G. C. Wong, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 11173.
- 5 D. D. Lasic, *Liposomes in Gene Delivery*, CRC Press, 2019.
- 6 A. J. Maheshwari, A. M. Sunol, E. Gonzalez, D. Endy and R. N. Zia, *Phys. Rev. Fluids*, 2019, **4**, 110506.
- 7 T. Vissers, A. T. Brown, N. Koumakis, A. Dawson, M. Hermes, J. Schwarz-Linek, A. B. Schofield, J. M. French, V. Koutsos, J. Arlt, V. A. Martinez and W. C. Poon, *Sci. Adv.*, 2018, **4**, 1170.
- 8 U. U. Ghosh, H. Ali, R. Ghosh and A. Kumar, *J. Colloid Interface Sci.*, 2021, **594**, 265–278.
- 9 S. Ikeda and K. Nishinari, *Biomacromolecules*, 2000, **1**, 757–763.
- 10 F. Zhang, M. W. Skoda, R. M. Jacobs, R. A. Martin, C. M. Martin and F. Schreiber, *J. Phys. Chem. B*, 2007, **111**, 251–259.
- 11 A. Stradner, H. Sedgwick, F. Cardinaux, W. C. Poon, S. U. Egelhaaf and P. Schurtenberger, *Nature*, 2004, **432**, 492–495.
- 12 E. J. Yearley, I. E. Zarraga, S. J. Shire, T. M. Scherer, Y. Gokarn, N. J. Wagner and Y. Liu, *Biophys. J.*, 2013, **105**, 720–731.
- 13 A. Stradner and P. Schurtenberger, *Soft Matter*, 2020, **16**, 307–323.
- 14 L. Nicoud, M. Owczarz, P. Arosio and M. Morbidelli, *Biotechnol. J.*, 2015, **10**, 367–378.
- 15 H. P. Erickson, *Biol. Proced. Online*, 2009, **11**, 32.
- 16 B. Jachimska, M. Wasilewska and Z. Adamczyk, *Langmuir*, 2008, **24**, 6867–6872.
- 17 R. Tuinier and A. Brûlet, *Biomacromolecules*, 2003, **4**, 28–31.
- 18 C. Gögelein, G. Nägele, R. Tuinier, T. Gibaud, A. Stradner and P. Schurtenberger, *J. Chem. Phys.*, 2008, **129**, 085102.
- 19 M. A. Woldeyes, C. Calero-Rubio, E. M. Furst and C. J. Roberts, *J. Phys. Chem. B*, 2017, **121**, 4756–4767.
- 20 K. Baler, R. Michael, I. Szeleifer and G. A. Ameer, *Biomacromolecules*, 2014, **15**, 3625–3633.
- 21 R. L. Dimarco and S. C. Heilshorn, *Adv. Mater.*, 2012, **24**, 3923–3940.
- 22 X. Zhang, S. Jiang, T. Yan, X. Fan, F. Li, X. Yang, B. Ren, J. Xu and J. Liu, *Soft Matter*, 2019, **15**, 7583–7589.
- 23 Y. Sun and Y. Huang, *J. Mater. Chem. B*, 2016, **4**, 2768–2775.
- 24 C. Huerta-López and J. Alegre-Cebollada, *Nanomaterials*, 2021, **11**, 1656.
- 25 W. A. Petka, J. L. Harden, K. P. McGrath, D. Wirtz and D. A. Tirrell, *Science*, 1998, **281**, 389–392.
- 26 H. Li, N. Kong, B. Laver and J. Liu, *Small*, 2016, **12**, 973–987.
- 27 S. H. Arabi, B. Aghelnejad, C. Schwieger, A. Meister, A. Kerth and D. Hinderberger, *Biomater. Sci.*, 2018, **6**, 478–492.
- 28 S. Khanna, A. K. Singh, S. P. Behera and S. Gupta, *Mater. Sci. Eng., C*, 2021, **119**, 111590.
- 29 S. Ikeda and K. Nishinari, *Biopolymers*, 2001, **59**, 87–102.
- 30 J. Fang, A. Mehlich, N. Koga, J. Huang, R. Koga, X. Gao, C. Hu, C. Jin, M. Rief, J. Kast, D. Baker and H. Li, *Nat. Commun.*, 2013, **4**, 2974.
- 31 L. R. Khoury and I. Popa, *Nat. Commun.*, 2019, **10**, 1–9.
- 32 M. D. Hughes, B. S. Hanson, S. Cussons, N. Mahmoudi, D. J. Brockwell and L. Dougan, *ACS Nano*, 2021, **15**, 11296–11308.
- 33 K. Shmilovich and I. Popa, *Phys. Rev. Lett.*, 2018, **121**, 168101.
- 34 M. Djabourov, K. Nishinari and S. B. Ross-Murphy, *Physical Gels Biol. Synth. Polym.*, 2013, 256–286.



- 35 A. H. Clark and S. B. Ross-Murphy, *Mod. Biopolym. Sci.*, 2009, 1–27.
- 36 A. P. Minton, *Mol. Cell. Biochem.*, 1983, **55**, 119–140.
- 37 P. S. Sarangapani, S. D. Hudson, R. L. Jones, J. F. Douglas and J. A. Pathak, *Biophys. J.*, 2015, **108**, 724–737.
- 38 S. Lv, T. Bu, J. Kayser, A. Bausch and H. Li, *Acta Biomater.*, 2013, **9**, 6481–6491.
- 39 M. D. G. Hughes, S. Cussons, N. Mahmoudi, D. J. Brockwell and L. Dougan, *Soft Matter*, 2020, **16**, 6389–6399.
- 40 Y. Tang, H. Wang, S. Liu, L. Pu, X. Hu, J. Ding, G. Xu, W. Xu, S. Xiang and Z. Yuan, *Colloids Surf., B*, 2022, **220**, 112973.
- 41 H. Li, *Adv. NanoBiomed Res.*, 2021, **1**, 2100028.
- 42 B. S. Hanson and L. Dougan, *Macromolecules*, 2020, **53**, 7335–7345.
- 43 L. Fu, A. Haage, N. Kong, G. Tanentzapf and H. Li, *Chem. Commun.*, 2019, **55**, 5235–5238.
- 44 S. Haas, S. Körner, L. Zintel and J. Hubbuch, *Front. Bioeng. Biotechnol.*, 2022, **10**, 1006438.
- 45 M. Slawinski, M. Kaeek, Y. Rajmiel and L. R. Khoury, *Nano Lett.*, 2022, **22**, 6942–6950.
- 46 T. Hoffmann, K. M. Tych, M. L. Hughes, D. J. Brockwell and L. Dougan, *Phys. Chem. Chem. Phys.*, 2013, **15**, 15767.
- 47 C. Gögelein, G. Nägele, J. Buitenhuis, R. Tuinier and J. K. Dhont, *J. Chem. Phys.*, 2009, **130**, 1–15.
- 48 Y. Liu and R. B. Pandey, *J. Phys. II*, 1994, **4**, 865–872.
- 49 S. Jungblut, J.-O. Joswig and A. Eychmüller, *Phys. Chem. Chem. Phys.*, 2019, **21**, 5723–5729.
- 50 P. Meakin, *Phys. Scr.*, 1992, **46**, 295–331.
- 51 W. C. Poon and M. D. Haw, *Adv. Colloid Interface Sci.*, 1997, **73**, 71–126.
- 52 D. W. Schaefer, *MRS Bull.*, 1988, **13**, 22–27.
- 53 L. F. Van Heijkamp, I. M. De Schepper, M. Strobl, R. Hans Tromp, J. R. Heringa and W. G. Bouwman, *J. Phys. Chem. A*, 2010, **114**, 2412–2426.
- 54 T. van Vliet, in *Hydrocolloids – Part 1*, ed. K. Nishinari, Elsevier Science, 2000, pp. 367–377.
- 55 Y. Li, B. Xue and Y. Cao, *ACS Macro Lett.*, 2020, **9**, 512–524.
- 56 J. Opdam, V. F. Peters, H. H. Wensink and R. Tuinier, *J. Phys. Chem. Lett.*, 2023, **14**, 199–206.
- 57 M. P. Howard, Z. M. Sherman, A. N. Sreenivasan, S. A. Valenzuela, E. V. Anslyn, D. J. Milliron and T. M. Truskett, *J. Chem. Phys.*, 2021, **154**, 074901.
- 58 T. Kwon, T. A. Wilcoxson, D. J. Milliron and T. M. Truskett, *J. Chem. Phys.*, 2022, **157**, 184902.
- 59 A. Aufderhorst-Roberts, M. D. G. Hughes, A. Hare, D. A. Head, N. Kapur, D. J. Brockwell and L. Dougan, *Biomacromolecules*, 2020, **21**, 4253–4260.
- 60 Á. Pérez-Benito, C. Huerta-López, J. Alegre-Cebollada, J. M. García-Aznar and S. Hervas-Raluy, *J. Mech. Behav. Biomed. Mater.*, 2023, **138**, 105661.
- 61 M. Takahashi, K. Yokoyama and T. Masuda, *J. Chem. Phys.*, 1994, **101**, 798.
- 62 J. C. Gimel, D. Durand and T. Nicolai, *Phys. Rev. B: Condens. Matter Mater. Phys.*, 1995, **51**, 11348–11357.
- 63 C. M. Sorensen, *Aerosol Sci. Technol.*, 2011, **45**, 765–779.
- 64 P. J. Flory, *J. Am. Chem. Soc.*, 1941, **63**, 3083–3090.
- 65 D. Stauffer, A. Aharony and A. Aharony, *Introduction To Percolation Theory*, Taylor & Francis, 2nd edn, 1994.
- 66 A. Bunde, P. Heitjans, S. Indris, J. W. Kantelhardt and M. Ulrich, *Diffus. Fundamentals*, 2007, **6**, 1–17.
- 67 H. Tsurusawa, M. Leocmach, J. Russo and H. Tanaka, *Sci. Adv.*, 2019, **5**, 6090.
- 68 A. Zaccone, H. H. Winter, M. Siebenbürger, M. Siebenb and M. Ballauff, *J. Chem. Phys.*, 2014, **58**, 1219–1244.
- 69 F. Hausdorff, *Math. Ann.*, 1918, **79**, 157–179.
- 70 A. S. Besicovitch, *Math. Ann.*, 1935, **110**, 321–330.
- 71 G. B. So, H. R. So and G. G. Jin, *Pattern Recognit. Lett.*, 2017, **98**, 53–58.
- 72 K. C. Clarke, *Comput. Geosci.*, 1986, **12**, 713–722.
- 73 I. Pilgrim and R. P. Taylor, in *Fractal Analysis of Time-Series Data Sets: Methods and Challenges*, ed. S.-A. Ouafeul, IntechOpen, Rijeka, 2019, ch. 2.
- 74 F. Arand and J. Hesser, *Comput. Geosci.*, 2017, **101**, 28–37.
- 75 T. A. Witten and L. M. Sander, *Phys. Rev. B: Condens. Matter Mater. Phys.*, 1983, **27**, 5686–5697.
- 76 M. Kolb and R. Jullien, *J. Phys., Lett.*, 1984, **45**, 977–981.
- 77 A. Moncho-Jordá, G. Odriozola, F. Martínez-López, A. Schmitt and R. Hidalgo-Álvarez, *Eur. Phys. J. E: Soft Matter Biol. Phys.*, 2001, **5**, 471–480.
- 78 M. Lattuada, H. Wu, A. Hasmy and M. Morbidelli, *Langmuir*, 2003, **19**, 6312–6316.
- 79 S. C. Pencea and M. Dumitrascu, *Fractal Aspects of Materials*, Boston, Massachusetts, USA, 1995, p. 373.
- 80 A. Hasmy and R. Jullien, *J. Non-Cryst. Solids*, 1995, **186**, 342–348.
- 81 M. Lach-hab, A. E. González and E. Blaisten-Barojas, *Phys. Rev. E: Stat. Phys., Plasmas, Fluids, Relat. Interdiscip. Top.*, 1996, **54**, 5456–5462.
- 82 S. Lazzari, L. Nicoud, B. Jaquet, M. Lattuada and M. Morbidelli, *Adv. Colloid Interface Sci.*, 2016, **235**, 1–13.
- 83 J. E. Martin and D. Adolf, *Annu. Rev. Phys. Chem.*, 1991, **42**, 311–350.
- 84 M. Takahashi, K. Yokoyama, T. Masuda and T. Takigawa, *J. Chem. Phys.*, 1998, **101**, 798.
- 85 P. Ball, *Nat. Mater.*, 2022, **1**.
- 86 C. T. van Campenhout, D. N. Ten Napel, M. van Hecke and W. L. Noorduyn, *Proc. Natl. Acad. Sci. U. S. A.*, 2022, **119**, e2123156119.
- 87 L. Würthner, F. Brauns, G. Pawlik, J. Halatek, J. Kerssemakers, C. Dekker and E. Frey, *Proc. Natl. Acad. Sci. U. S. A.*, 2022, **119**, e2206888119.
- 88 D. Ashkin, R. A. Haber and J. B. Wachtman, *J. Am. Ceram. Soc.*, 1990, **73**, 3376–3381.
- 89 L. D. Gelb, A. L. Graham, A. M. Mertz and P. H. Koenig, *Phys. Fluids*, 2019, **31**, 21210.
- 90 S. Babu, J. C. Gimel and T. Nicolai, *J. Phys. Chem. B*, 2008, **112**, 743–748.
- 91 I. M. El-Sherbiny and M. H. Yacoub, *Global Cardiol. Sci. Practice*, 2013, **2013**, 38.
- 92 M. W. Tibbitt and K. S. Anseth, *Biotechnol. Bioeng.*, 2009, **103**, 655–663.



- 93 D. Fan, U. Staufer and A. Accardo, *Bioengineering*, 2019, **6**, 113.
- 94 A. Upadhyay, R. Kandi and C. P. Rao, *ACS Sustainable Chem. Eng.*, 2018, **6**, 3321–3330.
- 95 R. Nandi, A. Yucknovsky, M. M. Mazo and N. Amdursky, *J. Mater. Chem. B*, 2020, **8**, 6964–6974.
- 96 Y. H. Chiang, M. J. Wu, W. C. Hsu and T. M. Hu, *J. Mater. Chem. B*, 2020, **8**, 8830–8837.
- 97 N. Sanaeifar, K. Mäder and D. Hinderberger, *Pharmaceutics*, 2021, **13**, 1661.
- 98 N. Sanaeifar, K. Mäder and D. Hinderberger, *Int. J. Mol. Sci.*, 2022, **23**, 7352.
- 99 S. H. Oh, I. K. Park, J. M. Kim and J. H. Lee, *Biomaterials*, 2007, **28**, 1664–1671.
- 100 S. Yang, K. F. Leong, Z. Du and C. K. Chua, *Tissue Eng.*, 2001, **7**, 679–689.
- 101 L. H. Y. Grace and T. Y. Wah, Effect of Collagen Gel Structure on Fibroblast Phenotype|Journal of Emerging Investigators, 2012, <http://www.emerginginvestigators.org/2012/11/effect-of-collagen-gel-structure-on-fibroblast-phenotype/>.

