Synthesis and Physicochemical Characterization of Gellan Gels

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ABSTRACT: Thermally stable hydrogels have been prepared starting from aqueous gellan physical gels by partial self-cross-linking of the polysaccharide chains (formation of essentially interchain ester bonds, with no added bridging moieties) with the aid of the water-soluble 1-ethyl-3-[3-(dimethylammino)propyl]-carbodiimide (EDC). The structure of the hydrated networks—physicochemical gels—was analyzed on the basis of the results of small-angle X-ray scattering experiments. Here a molecular model, which is composed of a bundle of 48 gellan double helices aligned in parallel, was proposed to represent the ordered domains in the hydrogels. The rigid bundles formed by associated gellan double helices constitute the junction zones which sustain the overall gel structure displaying solidlike properties. Relatively large cavities supported by rigid bundles inside the gels absorb water quickly as indicated by kinetic water uptake data. A network structure formed by rigid microfibrils of associated gellan double helices connected by flexible joints of disentangled short gellan chains—in which most of the chemical cross-linking should have taken place—is consistent with the rheological and swelling behavior of the new polymeric materials.

1. Introduction

The production of biocompatible hydrogels is a fundamental step toward the formulation of materials suitable for different biomedical applications going from controlled drug release to cells scaffolding. In this context, novel synthetic routes were recently explored for the preparation of hydrogels based on carbohydrate polymers.[1−4]

In fact, many polysaccharides including chitosan, scleroglucan, and hyaluronan have been successfully employed in the preparation of chemically cross-linked hydrogels of potential biomedical interest. All the syntheses have been performed in water (the use of organic solvents being avoided throughout) via the pairing at random of initially free, single polysaccharide chains.

However, this extensively utilized, conventional type of chain pairing entails formation of inhomogeneous and irregularly meshed networks with a number of structural defects including dangling chains and loops. These features have quite naturally a bearing on the physicochemical properties of the networks and hence also on their biological performances.

To obtain hydrogels with a more defined, even though roughly, internal structure and, in particular, good mechanical properties we have pursued the cross-linking of a polysaccharide whose chains could be already engaged in local, partial order, i.e., a polysaccharide giving rise to physical gelation mediated by the partial association of double helical segments in dilute aqueous media. To this end, one biopolymer of choice is quite naturally gellan.[5−8]

Gellan is a microbial exopolysaccharide applied widely in the food industry as a thickener or gelling agent. Gellan in aqueous salt solutions yields physical gels whose properties are markedly influenced by the nature and concentration of added salts.

Gellan gels are considered to be the result of the association of double helical stretches which allow formation of ordered junction domains interconnected switchboardlike by unordered chain segments.[9] If chemical cross-linking can be introduced so to involve mainly the disordered part of the gellan network, the ensuing gel would be stabilized by both specific physical interactions (among double helical stretches) and by chemical bonds. The latter would then give a sort of permanent set to the ordered domains of the initial physical gel: since size and number of the ordered domains can be controlled by the physical gel setting conditions, we should be able to prepare physical/chemical hydrogels with a useful range of physical characteristics.

The results we wish to report here deal with a study of the chemical cross-linking of gellan chains in the physical-gel state performed in aqueous media by activating the carboxylate groups along the polysaccharide chains—the disordered, easily accessible chain sections connecting the junction zones, we presume—so as to form interchain ester bonds (self-cross-linked gels, i.e., with no added bridging moieties).[10] Such bonds may exert a constraint on the gellan chains forcing them to interact topologically and electrostatically with an enhanced stability of the supramolecular structure.

To prove the above hypothesis, the structure of the physical/chemical hydrogels has been examined by small-angle X-ray scattering. A molecular model was built by assuming the lateral association of gellan double helices, and the scattering profiles were calculated from the model. The comparison of the scattering profiles confirmed the stabilizing effect of self-cross-linking of the chains in the disordered regions on the ordered domain composed of associated double helices, in qualitative agreement with swelling and rheological data.

2. Experimental Section

2.1. Materials. Gellan physical gel was prepared from its aqueous solution by cooling. Here a prescribed amount of the commercial product Gelrite was weighed in an Erlenmeyer

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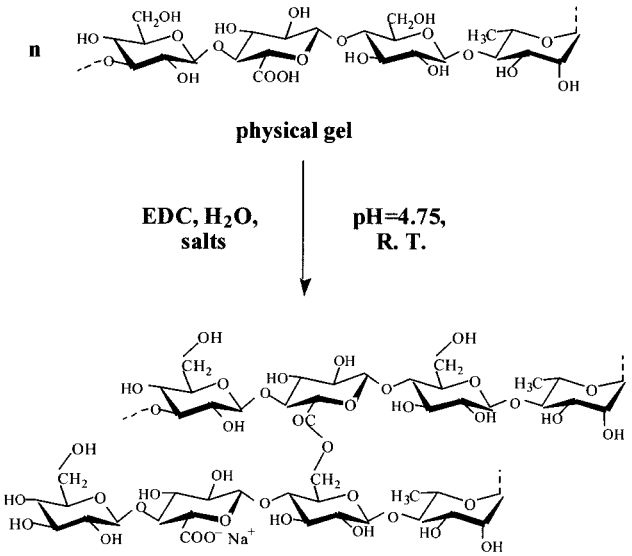

Figure 1. Scheme of the synthesis of gellan self-cross-linked physical/chemical hydrogels starting from gellan physical gel.

Table 1. Self-Cross-Linked Gellan Samples

sample code	c_p, % (w/v)[a]	c_s, % (w/w)[a]	DC[b]	$\Delta P/P_0$[c]
1	1	0.1	0.11	119.6
2	2	0.1	0.12	78.5
3	3	0.1	0.13	51.3

[a] Starting gellan (c_p) and NaCl (c_s) concentrations in physical gel [b] Stoichiometric degree of cross-linking, from HCl consumption during synthesis, defined as DC = (crosslinked (i.e., esterified) carboxylic groups of gellan)/(initial number of carboxylic groups). [c] Equilibrium weight ratio, $\Delta P/P_0 = (P - P_0)/P_0$, where P and P_0 are the weight of the swollen gel (after elimination of the residual air bubbles) and that of the dry starting material, respectively

flask. Water was added in the flask, the flask was sealed, and gellan was dissolved under stirring at 90 °C until any visible suspended particles disappeared. Then a known amount of NaCl (0.1% w/w) was added, and the hot solution was poured into a container and maintained at room temperature to set the gel with a desired shape. Once settled, the gel was introduced in the aqueous reaction medium, which contains an equivalent ratio $R \approx 2$ ($R \equiv$ equiv of EDC/equiv of carboxylic groups) of the activating agent, the water-soluble 1-ethyl-3-[3-(dimethylammino)propyl]carbodiimide (EDC). EDC is a zero-length cross-linker, since the activation of the carboxylic groups of gellan is followed by reaction with the hydroxyls of a neighboring polysaccharide chain to form ester linkages.[11,12] A constant pH value of 4.75 was maintained during the reaction at room temperature by addition of HCl until no further change in pH due to proton consumption was observed. The ensuing physical−chemical hydrogel was purified by dialysis against a large volume of distilled water at pH = 7 and then freeze-dried for further use. The synthetic route is summarized in the scheme of Figure 1.

Three hydrogels were prepared by changing the starting gellan concentrations c_p from 1% (w/v) to 3% (w/v) with the same salt concentration c_s = 0.1% (w/w) as summarized in Table 1. The degree of cross-linking (DC) was monitored by the proton uptake: a maximum value of about 0.1 was obtained for all samples (see Table 1).[13,14]

2.2. Small-Angle X-ray Scattering. The small-angle X-ray scattering was observed from the swollen gels with the SAXES installed at BL10C of the Photon Factory, Tsukuba, Japan. An incident X-ray from synchrotron radiation was monochromatized to $\lambda = 0.149$ nm with a double-crystal monochromator and focused at the position of the detector with a bent focusing mirror. The intensity of scattered X-ray was measured by a one-dimensional position sensitive proportional counter (the effective length 190 mm) positioned at the distance of about 1 m from the sample holder. The exact camera length was calibrated by the diffraction peaks of collagen fiber. The observed range was from $q = 0.2$ nm^{-1} to $q = 4.5$ nm^{-1} where q denotes the magnitude of the scattering vector defined by $q \equiv (4\pi/\lambda)\sin(\theta/2)$ with λ and θ being the wavelength of the incident beam and the scattering angle, respectively.

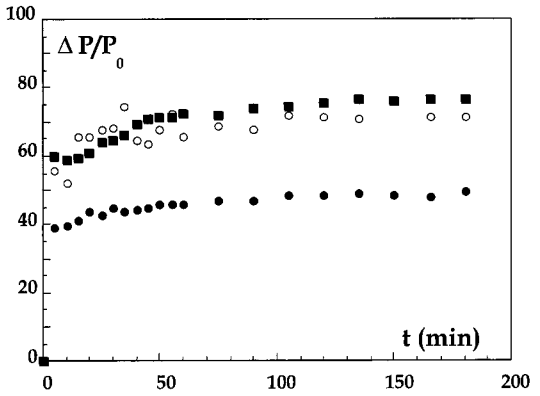

Figure 2. Weight ratio, $\Delta P/P_0 = (P - P_0)/P_0$, of gellan self-cross-linked networks in water at pH 7 (see Table 1) as a function of time. P and P_0 are the weight of the swollen gel and that of the dry starting material, respectively. Key: filled circles, sample 1; filled squares, sample 2; open circles, sample 3.

2.3. Swelling Experiments. Freeze-dried hydrogels of gellan were swelled in degassed water at pH = 7. The weight was recorded after eliminating the excess water around samples by gently drying with a kleenex. The weight ratio increase, $\Delta P/P_0 = (P - P_0)/P_0$, was evaluated as a function of time until no further change occurred (P and P_0 are the weight of the swollen gel and that of the dry starting material, respectively). The residual air bubbles trapped in the gels were eliminated under vacuum. The equilibrium weight ratios were evaluated from the weights of freeze-dried gels and swollen hydrogels as summarized in Table 1.

2.4. Rheological Characterization. Rheological experiments have been carried out using the parallel plate geometry (20 mm diameter, steel) of a Bohlin CS10 stress controlled rheometer. Sandpaper has been glued with cyanoacrylic glue onto each plate surface in order to avoid slippage of samples. Equilibrium swelling conditions have been maintained during rheological measurements by adding water at pH = 7 on the lower plate of the geometry until the entire free lateral surface of hydrogels have been completely wetted by the liquid.

Gap setting optimizations have been undertaken according to the procedure described elsewhere.[15]

Gels were subjected to the stress sweep experiments to obtain the nominal deformation inside the linearity range of mechanical moduli. Creep tests were also conducted before each measurement in order to ascertain the absence of any viscous effects. Storage and loss moduli, G' and G'', were determined as a function of the applied frequency in the range 0.01−10 Hz at 20 °C with a nominal deformation of 2×10^{-4}.[16] The temperature dependences of G' and G'' were observed at 0.1 Hz frequency and with a nominal deformation of 2×10^{-4} in the range 10−60 °C (1 °C/min heating rate).

3. Results and Discussion

3.1. Swelling Experiments. The results of swelling experiments are shown in Figure 2. The pattern obtained points out a limited but very rapid uptake of water by the gel during the initial 5 min. Visual inspection of the samples also show no appreciable volume increase.

Given this fact, the hypothesis of a sponge like structure with “rigid” supramolecular architecture appears to be plausible. The term rigid is used in the sense

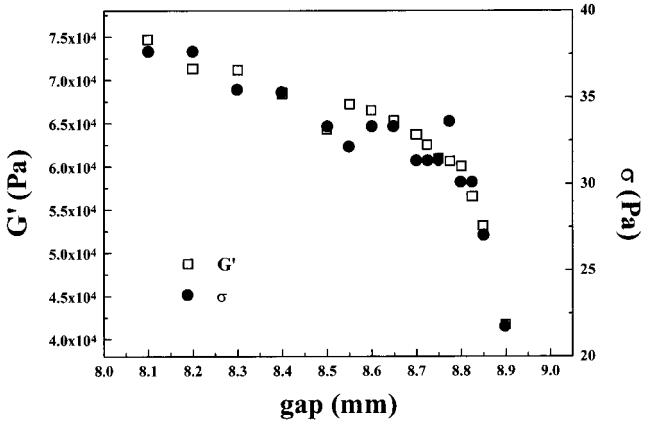

Figure 3. Storage shear modulus G' (□) and shear stress σ (●) as a function of the gap between parallel plate geometry for sample 3. (T = 20 °C; ν = 0.1 Hz; $\gamma_{max} = 1 \times 10^{-3}$.)

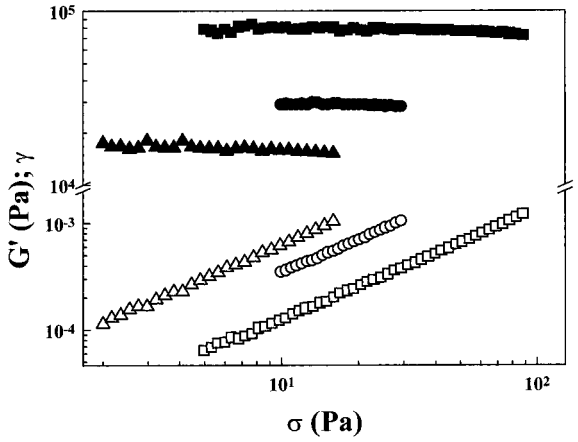

Figure 4. Shear stress (σ) sweep behavior of gellan physical/chemical hydrogels. (T = 20 °C; ν = 0.1 Hz; $\gamma_{max} = 1 \times 10^{-3}$.) Key: (▲) sample 1; (●) sample 2; (■) sample 3; full symbols, storage shear modulus G'; open symbols, nominal deformation γ.

of a structure able neither to rearrange and collapse nor to undergo visible volume variation due to solvent matrix interaction effects. The values of equilibrium weight ratios obtained (Table 1) are low for all the three different gels, with no significant final difference in order of magnitude, confirming a highly cross-linked gel, in which chemical zero-length bridges (interchain ester bonds) impose a topological constrain on the melting of double helices.

3.2. Rheological Characterization. Disks of the equilibrium swollen hydrogels were carefully put on the sandpaper glued onto the lower plate of the geometry. Then the upper plate was lowered until a good contact could be obtained. Since the sandpaper had to be pushed into the inhomogeneous surface of the gels while avoiding excessive compression, gap setting was a critical step to optimize rheological measurements. So, the gap was reduced with steps of 50 μm, and the final adopted value was evaluated from the profile of the applied stress (Figure 3). No slippage effect was detected during the creep tests.

The overall behavior of hydrogels is that of a solidlike material. Stress sweep experiments (Figure 4) show that the storage modulus is almost independent of the applied stress and justifies the choice of the nominal deformation adopted in successive measurements of 2 $\times$ 10^{-4}, which is a value inside the range of linearity.

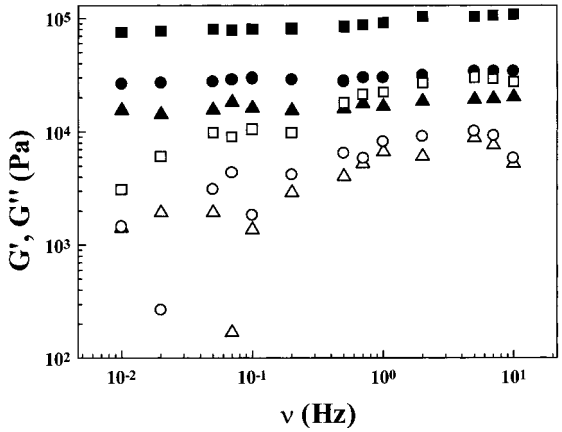

Figure 5. Storage (G') and viscous (G'') shear moduli behavior of gellan physical/chemical hydrogels as a function of applied frequency (ν). (T = 20 °C; $\gamma = 2 \times 10^{-4}$.) Key: (▲) sample 1; (●) sample 2; (■) sample 3; full symbols, G'; open symbols, G''.

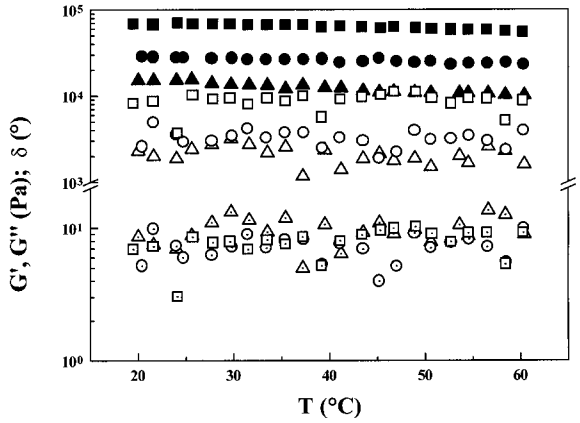

Figure 6. Shear storage moduli (G') and viscous moduli (G''), phase angle behavior (δ) as a function of temperature for gellan physical/chemical hydrogels. (ν = 0.1 Hz; $\gamma = 2 \times 10^{-4}$). Key: (▲) sample 1; (●) sample 2; (■) sample 3; full symbols: G'; open symbols, G''; dotted symbols, phase angle δ.

Dynamic−mechanical spectra (Figure 5) are typical of strong gels, and the moduli show little or no dependence on the applied frequency. Since measurements were conducted in an "open" system, the temperature dependence of shear moduli was investigated only in the range from 20 to 60 °C, to avoid internal evaporation of the solvent (Figure 6). All samples showed constant values of the shear moduli. The phase angle was monitored to check the occurrence of gel melting, and the recorded values confirm the absence of this phenomenon. The mechanical spectra of samples at 60 °C confirm the same behavior as those recorded at 20 °C, showing the shear moduli almost independent of frequency in the range 10^{-2}−10 Hz and a low phase angle (≈10°), evidence of the presence of a strong gel.

3.3. Small-Angle X-ray Scattering. The small-angle X-ray scattering from self-cross-linked gellan gels, prepared for starting polymer concentrations equal to 1%, 2%, and 3% w/v, leads to Kratky plots with a relatively sharp peak, which is typical of compact objects (see Figure 7 a−c). The scattering may be attributed mostly to the ordered domains within the gels, which have a higher electron density. The identical scattering profiles as presented in Figure 7 were observed up to 60 °C, indicating no change of the structure of the ordered domains for all samples observed.

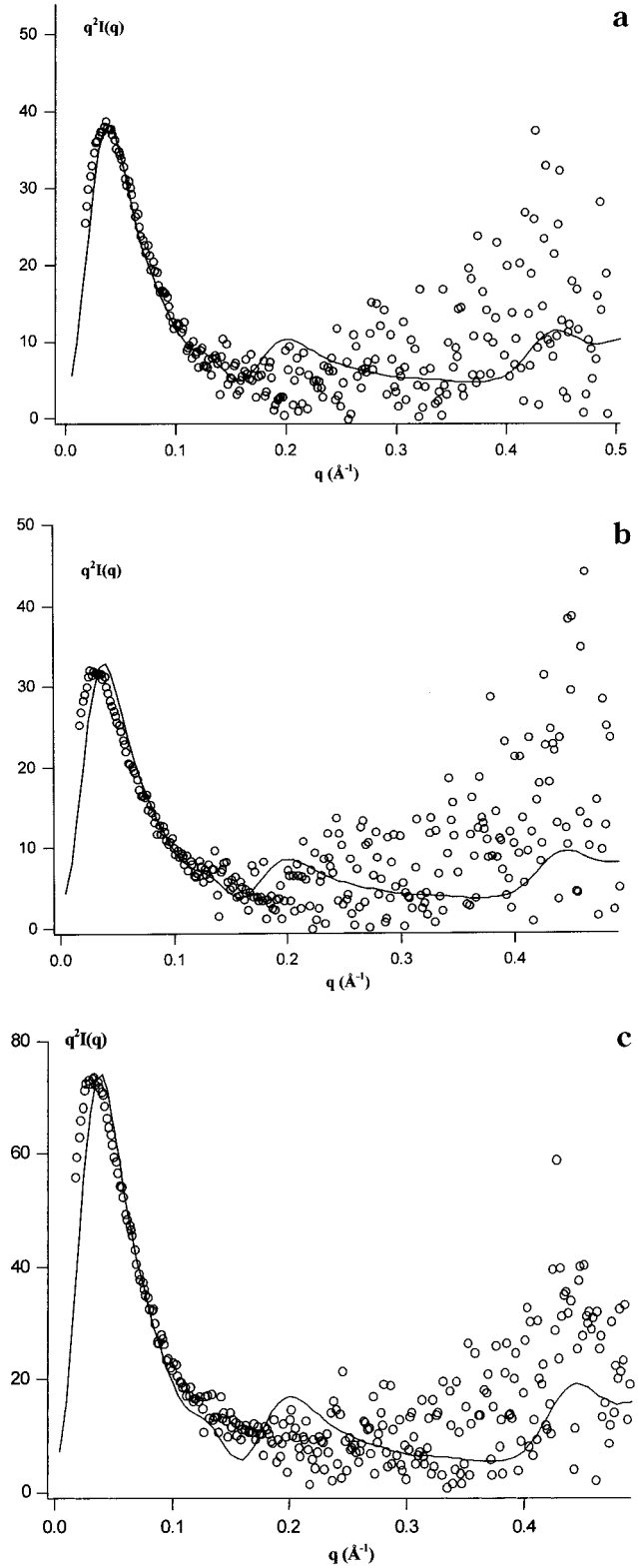

Figure 7. Small-angle X-ray scattering observed at 25 °C from self-cross-linked gellan gel (circles), and the calculated scattering profile from the molecular model of the ordered domain (solid lines). Key: (a−c) Self-cross-linked gellan hydrogels, starting concentration, c_p, in physical gels 1%, 2%, and 3%, respectively.

Gellan constitutes the ordered domain composed of multiple double helices aligned in parallel in gel.[9,17] The ordered domain functions as a junction zone in the gellan physical network. Since the chemical cross-linking is supposed to take place between the gellan chains not involved in the ordered domain, the chemical cross-links will stabilize the ordered structure by exerting a topological constraint on the domain as shown in Figure 8. Following the preceding practices,[9,17] we construct a molecular model for the ordered domain by packing gellan double helices according to the crystallographic data of Chandrasekaran et al.[18] Since the conventional Guinier plots for cross-section or thickness provided no satisfactory estimate for the corresponding parameters, the model was built on the assumption that the ordered domains would have a bulky shape. Figure 9 shows such a molecular model, which is composed of a bundle of 48 gellan double helices aligned in parallel. The molecular model was built so to yield the best-fit scattering profile to the observed SAXS profile from sample 1 (see Table 1). The bundle will be represented by a block of 12.48 nm × 4.05 nm × 8.46 nm in this instance and the bundle is considered—for computational simplicity—to represent an average of the real bundles having, of course, various shapes and sizes. The particle scattering factor $P(q)$ of the molecular model is calculated from the atomic coordinates by the Debye formula

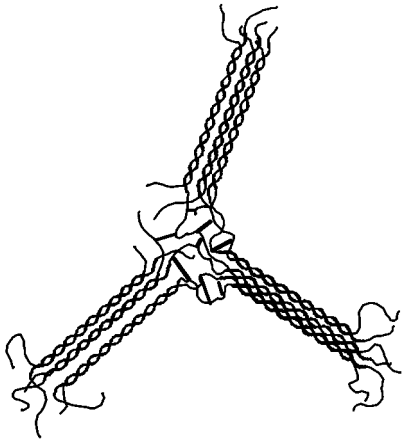

Figure 8. Schematic view of self-cross-linked gellan network structure, where heavy solid lines represent cross-linking points.

$$P(q) = \sum_{i=1}^{n} f_i^2 g_i^2(q) + 2\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} f_i f_j g_i(q) g_j(q) \frac{\sin(d_{ij}q)}{d_{ij}q} \quad (1)$$

where f_i and d_{ij} denote the atomic scattering weight of the atom i and the distance between the ith and jth atom, respectively. The form factor $g_i(q)$ for a single atom is assumed to be represented by the form factor of a rigid sphere having the radius equal to the van der Waals radius R_i of the ith atom. The values of 0.167 and 0.150 nm were employed for R_i of a carbon and an oxygen atom, respectively.

A solid line in Figure 7 is calculated by eq 1 with the atomic coordinates of the molecular model in Figure 9. Here the q_j^{-2}-proportional term is added to eq 1 in order to take into account the random spatial correlation among the ordered domains.[9] The agreement of the observed and calculated scattering profiles is satisfactory. Although each ordered domain is not necessarily composed of 48 double helices, a relatively large number of double helices are associated with constitute a domain, which is stabilized by chemical cross-links. Here the shift of the peak to a smaller q was observed in comparison with the calculated profile in sample 2

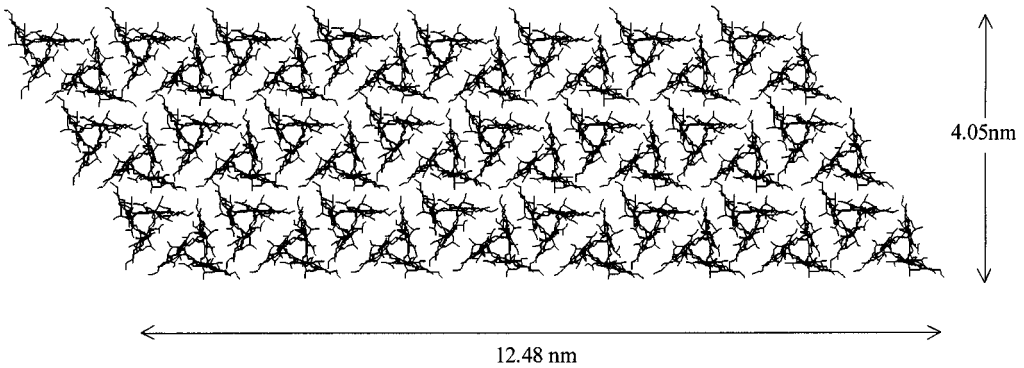

Figure 9. Top view of a molecular model for the ordered domain in gellan physical/chemical hydrogels. The model is composed of 48 double helices aligned in parallel.

and sample 3, indicating that increasing the initial gellan concentration larger ordered domains are formed. The network structure will thus be visualized as shown in Figure 8.

4. Conclusions

Stable hydrogels of an arbitrary shape can be prepared through self-cross-linking of gellan chains in the aqueous physical gel state by activating the carboxylic groups of the polysaccharide with the water-soluble 1-ethyl-3-[3-(dimethylammino)propyl]carbodiimide. We assume that the cross-links are introduced mainly, if not exclusively, in the disordered regions, which are in consequence thermally stabilized. The structure of the hydrogel was analyzed in terms of small-angle X-ray scattering results. Here a molecular model, which is composed of a bundle of 48 gellan double helices aligned in parallel, was proposed to represent the average ordered domain in hydrogel. The network formed by rigid walls of associated gellan double helices connected by flexible joints of disentangled short gellan chains yields a solidlike porous structure and accounts for the dynamic swelling behavior and rheological performances of the new physical/chemical hydrogels.

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