Supplementary Materials for

Molecular origin of the two-step mechanism of gellan aggregation

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Gellan hydrogels aging

To monitor aging effects, we performed rheological measurements on the same $G_{\text{pure}}$, $G_{\text{Na}}$, and $G_{\text{Ca}}$ samples at different times after the preparation. Figures S1A-C report the comparison of the storage and loss moduli $G'$ and $G''$ as a function of the shear strain $\gamma$ at two different times showing that no significant changes can be detected.

![Figure S1: Gellan hydrogels aging. Storage and loss moduli $G'$ and $G''$ as a function of the shear strain measured at different times after preparation (18 h and 43 h) at $T=25^\circ$C for gellan hydrogels at a polysaccharide concentration of 2 wt % with (A) pure gellan, (B) gellan with sodium chloride 27 mM, and (C) gellan with calcium acetate 2.5 mM.](image)

Validation of gellan force field

We have evaluated the soundness of our force field to reproduce the experimental aggregation of gellan through different approaches. First we have performed 10 additional independent simulation replicas of the systems $G_{\text{Na}}$ (5 wt %) and $G_{\text{Ca}}$ (5 wt %), for a total of eleven independent simulations for each system. Figures S2A-J and Figures S3A-J report the distribution of radius of gyration of each individual chain for the additional 10 simulation replicas of $G_{\text{Na}}$ (5 wt %) and $G_{\text{Ca}}$ (5 wt %), respectively (results of the eleventh simulation are shown in the main text of the manuscript).

Then we have compared simulations results for gellan with calcium acetate 0.05 M at a polysaccharide concentration of 5 wt % obtained using the atomic partial charges of our newly
developed force field with those obtained using the original in the CHARMM36 force field. The values of the atomic partial charges for the two force fields are summarized in Table S1. Figures S4A-J show the distribution of the radius of gyration ($P(R_{G})$) of each gellan chain for 10 independent replicas carried out using the CHARMM36 atomic partial charges. While for our newly developed force field different broad and sharp distributions of the radius of gyration, attributable to different chain conformations, are observed (Figures S3A-J), for CHARMM36 no variations are detected, nor formation of helix structures and extended aggregates (Figures S4A-J).

| Group                      | Atom  | CHARMM36 | new    |
|----------------------------|-------|----------|--------|
| β-D-glucose                | C1    | 0.290    | 0.298667 |
| β-D-glucose                | C2    | 0.140    | 0.066474 |
| β-D-glucose                | C3    | 0.140    | 0.119218 |
| β-D-glucose                | C4    | 0.090    | 0.200254 |
| β-D-glucose                | C5    | 0.110    | 0.229691 |
| β-D-glucose                | C6    | 0.050    | 0.203684 |
| β-D-glucose                | O2    | -0.650   | -0.621853 |
| β-D-glucose                | O3    | -0.360   | -0.437022 |
| β-D-glucose                | O4    | -0.650   | -0.715397 |
| β-D-glucose                | O5    | -0.400   | -0.546499 |
| β-D-glucose                | O6    | -0.650   | -0.704953 |
| β-D-glucose                | H1    | 0.090    | 0.095651 |
| β-D-glucose                | H2    | 0.090    | 0.099043 |
| β-D-glucose                | H3    | 0.090    | 0.099146 |
| β-D-glucose                | H4    | 0.090    | 0.058444 |
| β-D-glucose                | H5    | 0.090    | 0.036496 |
| β-D-glucose                | H61   | 0.090    | 0.054588 |
| β-D-glucose                | H62   | 0.090    | 0.054588 |
| β-D-glucose                | HO2   | 0.420    | 0.402987 |
| β-D-glucose                | HO4   | 0.420    | 0.439005 |
| β-D-glucose                | HO6   | 0.420    | 0.450311 |
| β-D-glucuronic acid        | C1    | 0.290    | 0.257354 |
| β-D-glucuronic acid        | C2    | 0.140    | 0.318595 |
| β-D-glucuronic acid        | C3    | 0.140    | 0.042422 |
| β-D-glucuronic acid        | C4    | 0.090    | 0.087925 |
| β-D-glucuronic acid | C5   | 0.110 | 0.042531 |
|---------------------|------|-------|----------|
| β-D-glucuronic acid | C6   | 0.520 | 0.875354 |
| β-D-glucuronic acid | O2   | -0.650 | -0.673471 |
| β-D-glucuronic acid | O3   | -0.650 | -0.718937 |
| β-D-glucuronic acid | O4   | -0.360 | -0.395148 |
| β-D-glucuronic acid | O5   | -0.400 | -0.443242 |
| β-D-glucuronic acid | O61  | -0.760 | -0.825435 |
| β-D-glucuronic acid | O62  | -0.760 | -0.825435 |
| β-D-glucuronic acid | H1   | 0.090 | 0.094327 |
| β-D-glucuronic acid | H2   | 0.090 | 0.112573 |
| β-D-glucuronic acid | H3   | 0.090 | 0.083692 |
| β-D-glucuronic acid | H4   | 0.090 | 0.105353 |
| β-D-glucuronic acid | H5   | 0.090 | 0.066513 |
| β-D-glucuronic acid | HO2  | 0.420 | 0.444322 |
| β-D-glucuronic acid | HO3  | 0.420 | 0.480174 |
| β-D-glucose        | C1   | 0.290 | 0.201172 |
| β-D-glucose        | C2   | 0.140 | 0.023633 |
| β-D-glucose        | C3   | 0.140 | 0.251145 |
| β-D-glucose        | C4   | 0.090 | 0.205766 |
| β-D-glucose        | C5   | 0.110 | -0.035713 |
| β-D-glucose        | C6   | 0.050 | 0.213947 |
| β-D-glucose        | O2   | -0.650 | -0.623962 |
| β-D-glucose        | O3   | -0.650 | -0.670336 |
| β-D-glucose        | O4   | -0.360 | -0.488503 |
| β-D-glucose        | O5   | -0.400 | -0.331659 |
| β-D-glucose        | O6   | -0.650 | -0.695593 |
| β-D-glucose        | H1   | 0.090 | 0.110912 |
| β-D-glucose        | H2   | 0.090 | 0.116235 |
| β-D-glucose        | H3   | 0.090 | 0.106824 |
| β-D-glucose        | H4   | 0.090 | 0.068972 |
| β-D-glucose        | H5   | 0.090 | 0.035713 |
| β-D-glucose        | H61  | 0.090 | 0.045148 |
| β-D-glucose        | H62  | 0.090 | 0.045148 |
| β-D-glucose        | HO2  | 0.420 | 0.437287 |
| β-D-glucose        | HO3  | 0.420 | 0.437362 |
| β-D-glucose        | HO6  | 0.420 | 0.429254 |
| α-L-rhamnose       | C1   | 0.290 | 0.091943 |
| α-L-rhamnose       | C2   | 0.140 | 0.164784 |
| α-L-rhamnose       | C3   | 0.140 | 0.245591 |
| α-L-rhamnose       | C4   | 0.090 | 0.028042 |
| α-L-rhamnose       | C5   | 0.110 | 0.305403 |
|α-L-rhamnose| C6  | -0.270 | -0.390280 |
|α-L-rhamnose| O2  | -0.650 | -0.682910 |
|α-L-rhamnose| O3  | -0.650 | -0.688366 |
|α-L-rhamnose| O4  | -0.360 | -0.388791 |
|α-L-rhamnose| O5  | -0.400 | -0.453004 |
|α-L-rhamnose| H1  | 0.090 | 0.172951  |
|α-L-rhamnose| H2  | 0.090 | 0.088685  |
|α-L-rhamnose| H3  | 0.090 | 0.042562  |
|α-L-rhamnose| H4  | 0.090 | 0.158716  |
|α-L-rhamnose| H5  | 0.090 | 0.086243  |
|α-L-rhamnose| H61 | 0.090 | 0.107484  |
|α-L-rhamnose| H62 | 0.090 | 0.107484  |
|α-L-rhamnose| H63 | 0.090 | 0.107484  |
|α-L-rhamnose| HO2 | 0.420 | 0.450668  |
|α-L-rhamnose| HO3 | 0.420 | 0.425346  |
|Methyl chain starting| CM  | 0.090 | 0.116232  |
|Methyl chain starting| HM1 | 0.090 | 0.038712  |
|Methyl chain starting| HM2 | 0.090 | 0.038712  |
|Methyl chain starting| HM3 | 0.090 | 0.038712  |
|Methyl chain ending  | CM  | -0.027 | -0.044241 |
|Methyl chain ending  | HM1 | 0.090 | 0.092539  |
|Methyl chain ending  | HM2 | 0.090 | 0.092539  |
|Methyl chain ending  | HM3 | 0.090 | 0.092539  |

Table S1: Atomic partial charges of the gellan repeating unit and the chain terminal groups. Glucuronic acid is in the ionized form.
Figure S2: **Reproducibility of MD simulations** $G_{Na}$ (5 wt %). Distribution of the radius of gyration ($P(R_G)$) of each gellan chain at 298 K calculated for gellan with sodium chloride 0.1 M at a polysaccharide concentration of 5 wt % for 10 independent replica (A-J). Data calculated for chain 1, 2, 3, 4, 5, and 6 are shown in cyan, red, blue, green, purple, and yellow, respectively.
Figure S3: Reproducibility of MD simulations $G_{Ca}$ (5 wt %). Distribution of the radius of gyration ($P(R_G)$) of each gellan chain at 298 K calculated for gellan with calcium acetate 0.05 M at a polysaccharide concentration of 5 wt % for 10 independent replica (A-J). Data calculated for chain 1, 2, 3, 4, 5, and 6 are shown in cyan, red, blue, green, purple, and yellow, respectively.
Figure S4: **MD simulations** $G_{Ca}$ (5 wt %) using CHARMM force field. Distribution of the radius of gyration ($P(R_G)$) of each gellan chain at 298 K calculated for gellan with calcium acetate 0.05 M at a polysaccharide concentration of 5 wt % for 10 independent replica (A-J) performed using CHARMM36 atomic partial charges. Data calculated for chain 1, 2, 3, 4, 5, and 6 are shown in cyan, red, blue, green, purple, and yellow, respectively.
Gellan inter-chains hydrogen bonds

Hydrogen bonding interactions occurring between gellan chains were investigated by evaluating the time evolution of the number of hydrogen bonds that each chain forms with all other chains. Figures S5A-I show the results obtained for each of the six gellan chains in the different investigated systems. In all conditions, the number of hydrogen bonds between associated gellan chains appears stable with time.

Figure S5: Gellan inter-chains hydrogen bonds. Time evolution of the total number of hydrogen bonds formed between a gellan chain with all the other chains from all-atom simulations at 298 K of pure gellan at concentration of 3 (A), 5 (D), and 10 wt % (G); gellan with sodium chloride 0.1 M at a polysaccharide concentration of 3 (B), 5 (E), and 10 wt % (H); and gellan with calcium acetate 0.05 M at a polysaccharide concentration of 3 (C), 5 (F), and 10 wt % (I). Data calculated for chain 1, 2, 3, 4, 5, and 6 are shown in cyan, red, blue, green, purple, and yellow, respectively.
Gellan chains conformation

We have investigated the conformation of gellan chains by first monitoring the time evolution of the radius of gyration of each polysaccharide chain, as displayed in Figures S6A-I. Aggregated gellan chains acquire rigid elongated conformations that exhibit a lower flexibility, as shown for the system \( G_{Ca} (5 \text{ wt} \%) \) (Figures S6F) in which the fluctuations of the radius of gyration for aggregated chains (such as chains cyan/yellow and red/green) are considerably reduced as compared to the other chains (chains purple and blue).

Then, we have further investigated the flexibility of gellan chains by analyzing the behavior of the dihedral angles of the glycosidic linkages between the monosaccharides composing the chains. To this aim, we have evaluated the number of transitions of all the dihedral angles of each gellan chain \( \Phi \), defined as H1-C1-O1-C4’, and \( \Psi \), defined as C1-O1-C4’-H4’. The occurrence of a dihedral transition was related to a dihedral angle change greater than 120°. The sequence of glycosidic dihedral angles was defined as shown in the Scheme S7. Figures S8A-I and Figures S9A-I summarize the results obtained for the dihedral angle \( \Phi \) and \( \Psi \), respectively. These findings show that the rotation of the dihedral angle \( \Phi \) is quite restricted, independently on the polysaccharide concentration, presence of salt, or aggregation state of the chain. Moreover, for the dihedral angle \( \Psi \), transitions are mostly detected for glycosidic linkages to glucose residues and are hindered for associated gellan chains, determining an increase of stiffness.
Figure S6: **Gellan chain flexibility.** Time evolution of the radius of gyration of each gellan chain at 298 K calculated for pure gellan at concentration of 3 (A), 5 (D), and 10 wt % (G); gellan with sodium chloride 0.1 M at a polysaccharide concentration of 3 (B), 5 (E), and 10 wt % (H); and gellan with calcium acetate 0.05 M at a polysaccharide concentration of 3 (C), 5 (F), and 10 wt % (I). Data calculated for chain 1, 2, 3, 4, 5, and 6 are shown in cyan, red, blue, green, purple, and yellow, respectively.
Figure S7: **Gellan dihedral angles.** Schematic representation of the sequence of the glycosidic dihedral angles $\Phi$ (defined as H1-C1-O1-C4') and $\Psi$ (defined as C1-O1-C4'-H4') in a single gellan chain.

Figure S8: **Glycosidic linkage $\Phi$.** Number of dihedral transitions of the glycosidic dihedral angle $\Phi$ defined as H1-C1-O1-C4' calculated over 100 ns for pure gellan at concentration of 3 (A), 5 (D), and 10 wt % (G); gellan with sodium chloride 0.1 M at a polysaccharide concentration of 3 (B), 5 (E), and 10 wt % (H); and gellan with calcium acetate 0.05 M at a polysaccharide concentration of 3 (C), 5 (F), and 10 wt % (I). Data calculated for chain 1, 2, 3, 4, 5, and 6 are shown in cyan, red, blue, green, purple, and yellow, respectively.
Figure S9: **Glycosidic linkage** $\Psi$. Number of dihedral transitions of the glycosidic dihedral angle $\Psi$ defined as C1-O1-C4'-H4' calculated over 100 ns for pure gellan at concentration of 3 (A), 5 (D), and 10 wt % (G); gellan with sodium chloride 0.1 M at a polysaccharide concentration of 3 (B), 5 (E), and 10 wt % (H); and gellan with calcium acetate 0.05 M at a polysaccharide concentration of 3 (C), 5 (F), and 10 wt % (I). Data calculated for chain 1, 2, 3, 4, 5, and 6 are shown in cyan, red, blue, green, purple, and yellow, respectively.
Electrostatic interactions

To evaluate the importance of electrostatic interactions, the contributions of the electrostatic, Lennard-Jones, and total energy have been calculated for the gellan simulations at a concentration of 5 wt %, as reported in Table S2. The main contribution to the total energy is associated to electrostatic interactions, specifically short range interactions, which is about one order of magnitude greater than all other terms, including Lennard-Jones interactions. It is noteworthy that the difference between the total energies of $G_{Ca}$ (5 wt %) and $G_{Na}$ (5 wt %) systems is very similar to the difference between the corresponding electrostatic energies. This finding correlates the higher structuring effect of calcium ions, as compared to that of sodium ions, to more efficient electrostatic interactions. Thus, the direct comparison of the different energy terms suggests that electrostatic interactions play a dominant contribution in the force field.

|                  | Electrostatic energy (kJmol$^{-1}$) | Lennard-Jones energy (kJmol$^{-1}$) | Total energy (kJmol$^{-1}$) |
|------------------|-------------------------------------|-------------------------------------|-----------------------------|
| $G_{pure}$ 5%wt  | -782000 (±13)                       | 79915 (±5)                          | -549132 (±3)                |
| $G_{Na}$ 5%wt    | -808600 (±40)                       | 81180 (±40)                         | -574700 (±10)               |
| $G_{Ca}$ 5%wt    | -829300 (±360)                      | 81980 (±60)                         | -594000 (±260)              |

Table S2: Electrostatic, Lennard-Jones, and total energy calculated for the simulations of gellan at a concentration of 5 wt %. For $G_{Na}$ and $G_{Ca}$ data are averaged over 11 replicas. The total energy is the sum of the electrostatic, Lennard-Jones, kinetic, and bonded interactions contributions.