ABSTRACT: Understanding spatiotemporal organization in bacteria under an external AC electric field is not only very interesting from a perspective of studying assembly and disassembly in a model biofilm but also provides insight into the intricate role of anisotropic interaction with bacterial dynamics that can generate interesting complex structures. In the current study, using confocal microscopy, we demonstrate such complex assemblies of monodisperse tetrad clusters of Micrococcus luteus, an environmental bacterium synthesized under a controlled growth condition. These clusters under the AC field produce a range of interesting structures such as chains, double helix, and bundles, which are instantaneously reversible when the field is switched off. Our studies can provide important insights into the natural organization of the clustered bacterium (with relevance in biofilm-like states) and generate strategies for biomaterial fabrication with a switchable functionality.

INTRODUCTION

Self-assembly is ubiquitous in both materials science and living cells. From materials science, we already know that different types of structural assemblies can be achieved through a unique control of particle shape, size, and interaction. So far, the majority of the past studies on self- and directed assemblies have been carried out using different types of uniform-sized spherical-shaped colloidal particles as successful model systems. In recent years, there is growing interest to use nonspherical-shaped and cluster type colloidal particles as building blocks to study complex assemblies useful in fabricating functional materials. Especially, clustered colloids are very interesting as they can be viewed as colloidal molecules. If spherical colloids can be modeled as atoms, then controlled aggregation of colloids into defined clusters can be considered as colloidal molecules. Molecular interactions and complex molecular assembly can be probed through the colloidal molecule approach using cluster type particles as building blocks.

On the other hand, addressing a biological problem using a bacterium as the model colloidal assembly and disassembly approach is also very interesting. Bacterial association and assembly play an important role in many infectious diseases as well as in the presence of matrix components (EPS) such as DNA, proteins, carbohydrates, and lipids. These EPS components generate isotropic and anisotropic interactions and enable bacterial particles to undergo complex assemblies. Structural complexities in bacterial biofilms can vary from bacteria to bacteria depending upon their complexity in shape, size, and metabolic properties. The viscoelastic property of the biofilm exhibits a viscous as well as an elastic response. So far, the physical interactions between bacteria in the presence of matrix components resulting in complex assemblies are not very well understood. Only a few studies exist based on the colloidal assembly analogy.

In these studies, it has been observed that the bacteria exhibit liquid crystalline structures in the presence of large-molecular-weight EPS substances like DNA, which act as a template and produce anisotropic elastic forces among bacteria. Template-driven strategies are not reversible and are extremely difficult to control for the study of assembly and disassembly. Templates forming chemical moieties are also

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usually toxic or non-biocompatible. This limits the ability to engineer bacteria into living materials with potential applications in biosensing and biomedicine. In this context, AC electric field induced anisotropic dipolar interaction is a novel method. It has been extensively used in the past to study template-free assembly and disassembly in many different types of colloidal systems, as well as in living systems such as bacteria, virus, and algae. Since bacteria can be considered as complex dielectric particles with a significant difference in their dielectric constant compared to the solvent (water), their response to an electric field is very rapid. The induced polarization causes individual bacteria to aggregate into formation of large structures aligned along the field direction. Due to the formation of these field-induced structures, the suspension exhibits a dramatic change of viscosity by several orders of magnitude. This property mimics that of an electrorheological fluid. Through the external AC field approach, high cell density equivalent to that present in a typical biofilm or biomaterial-like bacterial state can be generated rapidly from a very low-density bacterial suspension. Our recent studies on rod-shaped bacteria have already demonstrated interesting types of liquid crystalline structural organizations under an external AC electric field. Hence, we strongly believe that studying self-assembly of the clustered bacterium as the model colloid under an external AC electric field is interesting not only from a materials science view but also from a biological perspective.

In the current study, we have chosen *M. luteus* (ML) as a model for the clustered bacterium. *M. luteus* is a well-established model for studying biofilms and for bioremediations. Although it is known that *M. luteus* naturally occurs in symmetric dyads, tetrads, and octads, no detailed studies are reported in a bulk suspension under controlled growth conditions. Consecutive cell divisions without separation produce dyads, tetrads, and other clustered *M. luteus* structures. The first report of this process of consecutive cell divisions in *M. luteus* was in 1978, but the phenomenon mediating it was identified very recently in 2019. We observed that especially tetrad-shaped clusters are quite stable and common in the stationary phase of *M. luteus* as we verified from time-dependent growth studies. Hence, our AC electric field studies have been carried out using *M. luteus* bacterial tetrads. Our studies demonstrate that bacterial tetrads form instantaneously various types of field-induced reversible structures such as linear chain, bundle/columnar, and ribbon-like helix. It establishes a clustered colloidal model system to simulate and understand molecular structures and macromolecular behavior at easily observable length- and timescales using confocal microscopy. Lastly, strict control over structural assembly can be kept by varying field strength that demonstrates both a novel biomaterial fabrication strategy and a means to understand complex aggregation processes in bacterial clusters.

## RESULTS AND DISCUSSION

### Zero-Field Structural Ordering and Dynamics

*M. luteus* was grown under controlled growth conditions, which are typically used for a bacterial culture (see Experimental Methods). The growth curve was measured by a UV-vis spectrometer (Cary60, Agilent, USA) at a wavelength $\lambda = 600$ nm (see Figure 1A). Bacterial growth curves are often used to follow a sigmoidal profile (S-shape) where the bacterial growth rates are low at the beginning known as the lag phase, growth is exponential in the intermediate period known as the log phase, and growth slows down in the final period known as the stationary phase. Similar sigmoid profiles have been often seen in the case of nucleation and growth studies and phase transformation studies in different types of materials. This sigmoid curve is very well described by an exponential model of type $G(t) \sim [1 - \exp(-Kt^\alpha)]$, where $K$ and $\alpha$ are the rate of growth and the exponent denoting the dimensionality factor in two dimensions and 4 in three dimensions. Our sigmoid curve obtained from the bacterial growth characterization in bulk (three dimensions) is found to be in very good agreement with the exponential growth model. From the fitting, $K$ and $\alpha$ were found to be $3.4 \times 10^{-6}$ h$^{-1}$ and 4.4, respectively.

*M. luteus* undergoing *V snapping* division lengths to form poles (division sites) along one axis and segregates chromosomes. The cell division process of *M. luteus* is schematically shown in the inset of Figure 1. Two daughter cells are arranged around newly formed poles, which are held in a dynamic equilibrium until the last instant. When this dynamic equilibrium is breached by mechanical crack propagation, extremely rapid (millisecond) daughter cell separation occurs. This process is distinct from gradual enzymatically driven cell wall remodeling and division that is widely described in rod-shaped model bacteria (*Escherichia coli* and *Bacillus subtilis*). Rapid daughter cell separation produces dumbbell-shaped dyad bacterial clusters with close synchronization. Further, synchronized division of dyad clusters produces *M. luteus* tetrads from dyads without traversing through a triad stage.

We have collected cultures at different time points of the growth phase to look into structural ordering through confocal microscopy. *M. luteus* shows distinct structural associations in...
the lag, log, and stationary phases (see images in Figure 1B). M. luteus initially arranges into linked assemblies of two bacteria like a dumbbell shape around a 0–6 h growth period (lag phase to early log phase). As the bacterial cell division proceeds, bacteria divide in the form of tetrad clusters. The intermediate phase (from 6 h to late log phase, 20 h) contains mixtures of both dyads and tetrads as seen from the CLSM images (Figure 1B). In the stationary phase, majority of bacteria are in the form of tetrad clusters. It should be noted that these dyad and tetrad clusters are quite stable when exposed to shaking and even under low-frequency sonication. A closer look into the dimension of these clusters (see the schematics in Figure 1C) suggests that the center-to-center distance in dyad bacteria is slightly less than the diameter of single spherical bacteria due to the occurrence of deformation at the interface of cell division. Tetrad bacterial clusters show a cuboid-like shape (see the CLSM image in Figure 1B).

In the next step, we studied the dynamics of dyads and tetrads using angle-dependent dynamic light scattering (DLS) at very dilute concentration at room temperature (20 °C) in order to measure the translational diffusion coefficient. Their hydrodynamic radius ($R_h$) could be determined from these experiments (Figure 2). The experimental method and their analysis are detailed in the Experimental Methods section. Since the measured hydrodynamics radii are based on a sphere model using the Stokes–Einstein diffusion equation, we have approximated the volume of dyad (as cylinder shape) to an equivalent volume of a sphere. From this approximation, the theoretically calculated equivalent radii for the dyad and tetrad are found to be in close agreement with their measured hydrodynamic radii. It should be noted that, under the spherical approximation, the average hydrodynamic radii of the dyad and tetrad are found to be almost similar and close to 1.75 micron.

**AC-Field-Induced Structural Ordering and Dynamics.**

AC field studies are carried out on the purified bacterial sample collected from the stationary phase at 36 h, where the majority of bacteria are in tetrad form. The bacterial samples were purified by washing several times with Milli-Q water by centrifugation at 3000 rpm in order to remove the extra polymeric substances and salt contents. Further deionization was carried out by adding ionic-exchange resins to the bacterial suspension. Deionization leads to an increase in the surface charges of bacteria, which is confirmed by ζ-potential measurement. Under this deionized state, bacteria cells have an effective ζ potential of −29 mV. The electric field geometry implemented is shown in Figure 2B, where the electric field is applied in the XY plane, the same as the image plane, and the gap between the electrodes is kept at 1.2 mm. These field studies are carried out at room temperature ($T = 20 °C$) using confocal fluorescence microscopy (Leica, Germany).

In the first step, we have carried out a frequency-dependent study at constant electric field strength ($E = 0.01 \text{ V}_{\text{rms}}/\mu\text{m}$) in order to find out the frequency regimes where the field-induced structure formations are stronger. In the MHz-range frequency regime, tetrad bacteria form elongated chain-like structures along the electric field direction due to strong dipolar interaction (see the CLSM images in Figure 3). These structures do not disperse with decreasing frequency until 500 kHz. Further lowering the frequency, long chains start to melt around 200 kHz. The decrease in length as well as width of the...
chain can be clearly observed in the CLSM image at a frequency of 100 kHz (see Figure 3). In the low-frequency regime (10–1 kHz), the dipolar interactions further weaken, and the short chains also start to melt around 10 kHz. At 2 kHz, almost all chains are found to be melted. What remain are single tetrad bacteria clusters oriented along the field direction along with very few chains containing 2–3 tetrads.

To understand the frequency-dependent polarization behavior of bacterial tetrad, it is essential to determine the polarizability of bacterial tetrads through dielectric spectroscopy and find the strength of induced dipolar interaction. This study has been planned as an extension of this work at a later stage. So far, the existing polarization theories are only for nonclustered bacteria. Based on this existing polarization theory of AC field, we can only interpret our results qualitatively. In a deionized suspension, bacteria have surface charge, which is already evident from ζ-potential measurement in dilute concentration. This surface charge contributes to the double-layer potential. In theory, bacterial cells are often considered as complex dielectric particles, where different components of bacteria (such as cell membrane, cytoplasm) contribute to the total polarization. It is possible that double-layer polarization occurs in the low-frequency regime (~kHz) where the surface charge of bacteria plays an important role. In the high-frequency regime (~MHz), major components of the bacterial cell contribute to dielectric polarization. In our case, there is a possibility that the magnitude of the effective dipole moment arising from the dielectric polarization is much higher than the double-layer polarization (see the schematic in Figure 3). Hence, the dipolar interaction in the MHz frequency range is stronger than interaction in the kHz range and is responsible for these elongated structures. The structures get weaken as we decrease the frequency to the kHz range due to weakening of the dipolar interaction. So, we fix the frequency in the MHz range (~3 MHz) and vary the voltage in order to look into the details of the structural organizations as a function of electric field strength.

Figure 4 shows 2D confocal images of a deionized bacterial suspension containing tetrads (from the stationary state at 36 h) as a function of electric field strength, E (Vrms/μm). At E = 0, bacterial tetrads are diffusive due to Brownian motions and almost have no structural organization (see the 2D image at E = 0). Since the field experiments are carried out in bulk using a spacer of thickness 120 microns (≫ diameter of a single tetrad) and tetrads are also constantly undergoing Brownian motion, in the 2D view, the tetrads can sometimes look like a dyad or a sphere also. This will be clarified by looking at the time-series movies of both dyad and tetrads at E = 0 (see Movies S1 and S2). With increasing field strength, bacterial tetrads slowly start to orient along the field direction with their length parallel to the electric field (see the image at E = 0.0024 Vrms/μm). At E = 0.0035 Vrms/μm, the orientation of tetrads along E is clearly visible along with formation of short chains containing 2 or 3 tetrads. At this low field strength, there is always a competition between thermal fluctuation due to Brownian motion and the field-induced dipolar interaction. Hence, breaking and reformation of chains undergo instantly at the low electric field strength. Further, slightly increasing the field strength (E = 0.0059 Vrms/μm), chain length increases, and more tetrads are present in a chain (see Figure 4). At the same time, chains are stable against breaking and reformation. The structural organizations of bacterial tetrads at different field strengths are presented schematically in Figure 4.

In the high-field-strength regime, E = 0.008 to 0.01 Vrms/μm at a fixed frequency of 3 MHz, chain length of tetrads grows along the field direction due to strong dipolar interaction (see Figure 5A). At the same time, one-dimensional (1D) chains start to associate sideway (or laterally) to form two-
dimensional (2D) structures that mimic a double helix pattern (see Figure 5). Time-series studies demonstrate that, once these double-helix-like structures form, they do not dissociate unless the field is switched off (see Movie S3 and Figure SB). As a function of time, the double helix structure grows in width through association of more chains and forms a bundle type structure (see Figure SC).

Association of chains in the lateral direction is a very familiar phenomenon in colloidal systems under an external AC electric field.3-5 Chains that form along the field direction undergo constant fluctuation due to the intervening water medium. At any instant of time due to fluctuation, the chain looks like a wave structure with periodic maxima and minima (see the schematic in Figure SD). The attraction between the individual chains is the sum of individual pairwise dipole—dipole interaction between spheres in two chains.5 As a result, the dipolar interaction will be repulsive when the two chains are in-phase, and the interaction will be attractive at a short distance when two chains are out-of-phase. Under this attractive interaction, chains overlap and form aggregates, and the aggregate size increases as a function of time. Earlier studies on different types of dipolar spheres have shown that the aggregates of chains form well-defined ordered lattice structures with square symmetry in 2D (XY plane).5,8 However, in our case, we clearly see a helix type structure in the initial state of two-chain aggregation, which later on leads to bundle type containing multiple chains (see the schematic in Figure SD). The helix structure also undergoes similar fluctuation like an individual chain as it can be observed from Movie S3. In the later stage, we also observe that some of these large bundles span from one side of the electrode to the other side when the samples are kept for a longer period under constant field strength (see Figure 6).

Figure 6. 2D image of a helix/bundle of bacterial tetrads close to one of the electrodes, where the chain spanning is observed.

Although the exact mechanism for the formation of this helix structure is currently not known, we believe that the attractive/repulsive interaction coupled with the shape anisotropy and/or patchiness of the bacterial tetrad could be a possibility for this helix structure formation in bacterial tetrads under an external AC electric field. Some of the recent AC field studies using either Janus colloids or binary colloid mixtures (at different size ratios) have also shown a similar type of helix structure. Previously, long-range ordered helical lattice arrangements at nano- and mesoscales required templating, biomimeralization, or physical confinement and capillary force.7,42 Electric-field-driven assembly enables dynamic self-assembled micrometer-scale helices, which were not possible through previous methods. It also enables reversible iterative helix assembly and disassembly that increases the number of data points available for theoretical/simulation studies.

■ CONCLUSIONS

We have demonstrated assembly of bacteria into uniform-sized dyads and tetrad clusters under controlled growth conditions and further study the self-assembly of these tetrad clusters under the presence of an external AC electric field. The AC field produces interesting structures such as chains, double helix, and bundles, which can be tuned by varying the frequency (from MHz to kHz) and field strength (E = 0 to 0.01 V/μm) as well, and this process is also reversible. We strongly believe that these self-assembled structures from bacterial tetrads actuated by an external stimulus (AC field) could find use in fabrication of biomaterials with switchable functionalities. It can also provide a method for studying assembly and disassembly in future studies on bacterial biofilm models.

■ EXPERIMENTAL METHODS

**Bacterial Growth Curve.** *M. luteus* was procured from MTCC (strain no. 1538). Fresh liquid media were inoculated with overnight grown *M. luteus* culture at 1:100 dilution. Bacteria were cultured in a shaking incubator at a constant temperature of 30 °C and 150 rpm aeration in 200 mL of nutrient-rich Luria–Bertani broth without antibiotics. 1 mL of bacterial culture was removed at discrete time intervals. Growth curve progression was estimated by optical density measurement at 600 nm by a UV–vis spectrophotometer (Cary60). Simultaneously, a colony-forming unit per mL (cfu/mL) of culture was found out by plating serial dilutions on LB agar plates. These plates were incubated, and numbers of colonies were counted. For *M. luteus*, plates were incubated at 30 °C for 48 h. The growth curve with respect to time was plotted based on cfu/mL values as it provides viable cell numbers.

**Confocal Fluorescence Microscopy and Image Analysis.** A fixed volume (20 μL) of *M. luteus* cultures aliquoted at discrete time points that represent specific growth phases (lag, log, and stationary) was stained with the FITC dye. Dyed samples were deposited on a glass slide with a 120 μm adhesive spacer. A coverslip was placed over the deposited culture. The 120 μm spacer enables imaging of the bulk bacterial suspension without artifacts arising from wall effects.

Imaging was done with confocal laser scanning microscopy (TCS SP6, Leica Microsystems, Germany) at 63× magnification. Laser and filters were set for green fluorescent protein imaging (excitation 488 nm, emission 509 nm). Several images were taken at different positions for each sample. The average number of cells in each sample, motility (if any), Brownian dynamics, and clustering were evaluated.

**Dynamic Light Scattering.** A red laser of wavelength λ = 633 nm was used for this study. The intensity autocorrelation function g(2)(Q,t) is measured at different scattering angles θ (see Figure 2A). The field autocorrelation function g(1)(Q,t) is extracted using the Siegert relation as g(1)(Q,t) = 1 + i/2 Qf(t)Qf(t)i/2, where Q is the scattering vector and relate the scattering angle θ by Q = 4πn0sin(θ/2), where n0 is the refractive index of water. The field correlation function was analyzed by cumulant analysis using ln[g(1)(Q, i)] = −iΓt + μi/2, where Γ is the average decay rate and μ is the second-order cumulant coefficient and is related to the degree of polydispersity like μ/Γ2. The decay rate is directly related to the translational free diffusion coefficient by Γ = D0Q2 (see Figure 2B). From the
slope, we have calculated the translational free diffusion coefficient \( D_0 \). Finally, the hydrodynamic radius \( R_h \) was derived through the Stokes–Einstein equation, \( D_0 = k_BT/6\pi \eta R_h \) where \( k_B \) is the Boltzmann constant, \( T \) is absolute temperature, and \( \eta \) is the viscosity of the solvent.

**AC Electric Field Setup.** The AC electric field was applied by using a setup described in our previous paper.\(^{10}\) Briefly, a function generator and amplifier were used to provide the AC electric field of required strength and frequency. A bacterial suspension was held between two ITO coverslips for the parallel electric field and between a normal coverslip and etched ITO coverslips for the perpendicular electric field (Figure 2C). Bacteria were imaged with a confocal laser scanning microscope (Leica SP6 and Zeiss LSM S10). Data was analyzed with proprietary software and ImageJ.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04124.

Movie S1 represents the two-dimensional time-series movie of dyad bacteria at zero field (AVI)

Movie S2 represents the two-dimensional time-series movie of tetrad bacteria at zero field (AVI)

Movie S3 represents helical structures that formed when two chains overlap in the presence of an electric field. The helical structure dissociates when the field is switched off (AVI)

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**Notes**

The authors declare no competing financial interest.

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