The bacterial cell division protein, FtsZ, polymerizes to form a cytokinetic Z-ring at the midcell, which engineers bacterial cell division. Herein, we have examined the effects of an inorganic polyamine, cobalt hexamine trichloride, on the assembly of FtsZ in vitro. Cobalt hexamine trichloride strongly enhances FtsZ assembly, suppresses its GTPase activity and stabilizes FtsZ polymers. However, CoCl₂ and MnCl₂ have no detectable effect on the assembly of FtsZ in vitro. Interestingly, FtsZ is found to assemble into toroidal structures in the presence of low concentrations of cobalt hexamine trichloride. The Z-ring in bacterial cells appears to be a toroidal-like structure, suggesting that the use of cobalt hexamine trichloride may help to understand the assembly of FtsZ into toroidal structure. The toroidal structures may also serve as templates to build toroidal resonators for electrical and thermal applications.

Keywords: Bioinorganic chemistry, Toroidal condensation, Polymer assembly, Morphology, Polyamines, Inorganic polyamines, Cobalt hexamine trichloride, FtsZ, Z-ring

FtsZ is a bacterial cell division protein and a homolog of the well studied eukaryotic cell division protein, tubulin. FtsZ is the first protein known to assemble at the site of cell division and specifically form a circle at the centre of the cell. This structure known as “Z-ring” plays an important role in the constriction of the bacterial membrane leading to bacterial cytokinesis. The Z-ring assembly and disassembly takes place in collaboration with many other proteins and crowding agents. In our earlier work, two complimentary techniques, viz., transmission electron microscopy and small angle X-ray scattering were used to show that FtsZ assembles into different forms as a function of assembly conditions. Of the different configurations, viz., rod, ribbon, sheet and tube like structures that FtsZ assembled into, the tube structure formed in presence of DEAE-dextran was more interesting. The electron microscopy images showed the structures assuming toroid or ring like formations. These structures formed in vitro appear identical to the Z-ring formed inside the bacterial cell. The striking similarity between the toroids generated in vitro and the Z-ring in vivo suggests that FtsZ suprastructures assume specific formations that are suitable for the cellular processes they participate in.

DNA condensation has been shown to produce a variety of DNA structures (toroids, rod and spherical globules). Of all these, the toroidal structures have aroused great curiosity; the reason for such an interest being the most favorable morphology used by nature for high density DNA packaging in a cell. Cobalt hexamine trichloride is an inorganic polyamine known to induce toroidal DNA condensation. Its high stability in solution and toroidal morphology of DNA, which imitates the Z-ring of a bacterial cell, prompted us to choose cobalt hexamine trichloride for studies on FtsZ polymerization. The main purpose of this study is to understand the assembly dynamics of FtsZ that transform into toroidal structures, which is vital in understanding the assembly of FtsZ into Z-ring in the bacterial cell during cytokinesis.

In this study, the effects of cobalt hexamine trichloride on FtsZ assembly and polymer morphology have been investigated using an array of techniques. We found that FtsZ polymers condensed into toroids in the presence of cobalt hexamine trichloride. A mechanistic insight of the toroids assembly of FtsZ polymers is presented.

Materials and Methods
Piperazine-1,4-bis(2-etanesulfonic acid) (PIPS), isopropyl-β-D-thiogalactopyranoside (IPTG), glutamate and GTP used for the studies were obtained from Sigma. Cobalt hexamine chloride [Co(NH₃)₆]Cl₃ was...
procured from Himedia. All other chemicals were of analytical grade.

The recombinant *Escherichia coli* FtsZ was purified according to the previously described protocol using ammonium sulfate precipitation, followed by glutamate induced polymerization and cold induced depolymerization. Ammonium sulfate was removed using a desalting column (Bio-Gel P6 Gel, Bio-Rad) equilibrated in 25 mM Pipes pH 6.8. FtsZ concentration was determined by Bradford method and bovine serum albumin (BSA) was used as a standard. The purified protein was aliquoted and stored frozen at −80 °C.

**FtsZ polymerization by light scattering assay**

FtsZ (12 µM) in 25 mM PIPES, pH 6.8 containing 10 mM MgCl₂ (PM buffer) was incubated for 10 min in the absence and presence of 0.1 mM and 1 mM cobalt hexamine trichloride on ice. Immediately after adding 1 mM GTP, the reaction mixture was transferred to 37 °C and the kinetics of FtsZ polymerization was monitored by 90° light scattering for 15 min using a Jasco 6500 fluorescence spectrophotometer. Another set under similar conditions without the addition of GTP was also used. The light scattering signals of the samples were corrected by subtracting appropriate blanks. The excitation and emission wavelength was 500 nm.

**Measurement of FtsZ polymer mass by sedimentation assay**

FtsZ (6 µM) in PM buffer was incubated for 10 min in the absence and presence of 10, 20, 50, 100 and 200 µM cobalt hexamine trichloride on ice. Then, 1 mM GTP was added to the reaction mixture and polymerized at 37 °C for 15 min. The polymers were collected by centrifugation at 2,27,000 × g for 20 min at 30 °C. FtsZ concentration in the supernatant was measured by Bradford method using BSA as a standard. The amount of polymerized FtsZ was determined and the percent of total FtsZ pelleted in the absence and presence of different concentrations of the cobalt hexamine trichloride was calculated. Alternatively, the pellet was dissolved in 30 µl PIPES buffer containing 0.5 % SDS and analyzed by SDS-PAGE.

**Measurement of GTPase activity of FtsZ**

The production of inorganic phosphate during the FtsZ assembly after GTP hydrolysis was measured using a standard malachite green ammonium molybdate assay. FtsZ (6 µM) in PM buffer was incubated for 10 min on ice in the absence and presence of different concentrations of cobalt hexamine trichloride. Then, 1 mM GTP was added to each sample and immediately, the reaction mixtures were transferred to 37 °C. The hydrolysis reaction was quenched at specific time intervals by adding 10 % (v/v) 7 M perchloric acid and the quenched reaction mixtures were kept on ice. After collecting all samples, the quenched reaction mixtures were subsequently kept at 25 °C for 10 min. The samples were then incubated with freshly prepared solution containing 0.045 % malachite green (w/v), 4.2 % ammonium molybdate (w/v) and 0.02 % Triton X-100 (v/v) at 25 °C for 30 min and then the absorbance at 650 nm were recorded. All the readings were subtracted from the respective blanks without FtsZ.

**Stability of FtsZ polymers in the presence of cobalt hexamine trichloride**

FtsZ (25 µM) was polymerized in presence of 1 M glutamate, 5 mM MgCl₂ and 1 mM GTP at 37 °C for 20 min. The polymers formed were then diluted 20 times to reach a final FtsZ concentration of 1.25 µM in warm PIPES buffer containing 0, 0.1, 0.5 and 1 mM concentrations of cobalt hexamine trichloride and incubated at 37 °C for another 15 min. Polymers were sedimented at 2,27,000 × g for 20 min at 30 °C. The protein concentration in the supernatant was measured by Bradford method using BSA as a standard. The amount of polymerized FtsZ was determined and the percent of total FtsZ pelleted in the absence and presence of different concentrations of the cobalt hexamine trichloride was calculated.

**Visualization of FtsZ polymers under electron microscopy**

FtsZ (6 µM) in PM buffer was incubated for 10 min on ice in the absence and presence of different concentrations (30, 50, 100 µM and 1 mM) of cobalt hexamine trichloride. 1 mM GTP was added to the reaction milieu and FtsZ was polymerized at 37 °C for 15 min. The polymers were examined using a FEI Tecnai-G² 12 transmission electron microscope as described previously. Briefly, FtsZ polymeric suspension was placed on formvar carbon-coated copper grids and then blotted dry. The grids were subsequently subjected to negative staining by 1 % uranyl acetate solution and air-dried.
Results and Discussion

Effects of cobalt hexamine trichloride on the assembly kinetics of FtsZ

In the presence of GTP, cobalt hexamine trichloride enhanced the light scattering intensity of FtsZ assembly in a concentration dependent manner indicating that it promoted FtsZ assembly (Fig. 1a). The initial scattering profile indicated that cobalt hexamine trichloride facilitated the nucleation of FtsZ assembly. The light scattering intensity of FtsZ polymers after 15 min of assembly in the absence of cobalt hexamine trichloride was 6 A.U. while it was found to be 70 and 140 A.U. in the presence of 0.1 mM and 1 mM cobalt hexamine trichloride, respectively (Fig. 1a). The scattering intensity increased by 12 and 23 folds as compared to that of the control in the presence of 0.1 mM and 1 mM cobalt hexamine trichloride, respectively, suggesting that cobalt hexamine trichloride increased the polymeric mass of FtsZ and induced extensive bundling of polymers. If GTP was not added into the assembly milieu, cobalt hexamine trichloride could not promote the light scattering signal of FtsZ assembly (Fig. 1b). For example, in the absence of GTP, the light scattering intensity of FtsZ assembly after 15 min was found to be 1.5, 2.0 and 2.8 A.U without and with 0.1 and 1 mM cobalt hexamine trichloride, respectively (Fig. 1b). The results suggest that GTP is essential for cobalt hexamine trichloride induced assembly of FtsZ.

Interestingly, CoCl₂ and MnCl₂ did not promote FtsZ polymerization in the presence of GTP. The light scattering intensity at 15 min in the presence of 0.1 and 1 mM CoCl₂ and MnCl₂ was 6 A.U. (Fig. 2). Overall, these results indicate that the ligand (hexamine group) bound to the metal ion plays an important role in FtsZ polymerization rather than the metal ion unaccompanied.

Cobalt hexamine trichloride increases the sedimentable polymeric mass of FtsZ

FtsZ (6 µM) was polymerized with different concentrations of cobalt hexamine trichloride in the presence of 1 mM GTP at 37 °C and polymers were

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Fig. 1 — Effects of cobalt hexamine trichloride on the assembly of FtsZ. [FtsZ (12 µM) was polymerized in PM buffer at 37 °C in the presence (panel a) or absence (panel b) of 1 mM GTP. Traces in ‘panel a’ represent the kinetics of FtsZ assembly without (■) and with either 0.1 mM (●) or 1 mM (▲) cobalt hexamine trichloride in the presence of GTP. The traces in ‘panel b’ represent the kinetics of FtsZ assembly without (□) and with either 0.1 mM (○) or 1 mM (▲) cobalt hexamine trichloride in the absence of GTP. (Note: The difference in the scale on the Y-axis of panels a and b).]

Fig. 2 — Effects of CoCl₂ and MnCl₂ on the kinetics of FtsZ assembly. [FtsZ (12 µM) was polymerized in PM buffer and 1 mM GTP at 37 °C in the absence (■) and presence of CoCl₂ (0.1 mM (○) and 1 mM (▲)) and MnCl₂ (0.1 mM (●) and 1 mM (▲)). Note: The difference in the scale on the Y-axis compared to Fig. 1a].
collected through high speed sedimentation. Cobalt hexamine trichloride increased the sedimentable polymeric mass of FtsZ in a concentration dependent manner (Fig. 3). While 28 ± 3 % of the total FtsZ was pelleted in the absence of cobalt hexamine trichloride, 44 ± 5 %, 59 ± 1 %, 69 ± 3 %, 72 ± 4 % and 75 ± 3 % of the total FtsZ was sedimented in the presence of 10, 20, 50, 100 and 200 µM of cobalt hexamine trichloride, respectively (Fig. 3). The amount of sedimentable polymeric mass of FtsZ reached an optimal value around 200 µM of cobalt hexamine trichloride. Higher concentrations (> 200 µM) of cobalt hexamine trichloride did not increase the amount of polymerized FtsZ. However, 1 mM of cobalt hexamine trichloride did not increase in the light scattering signal indicating that cobalt hexamine trichloride induced bundling of FtsZ polymers and also altered the size and shape of the polymers.

**Cobalt hexamine trichloride inhibites the GTPase activity of FtsZ**

The dynamics of FtsZ is driven by hydrolysis of GTP\(^{37,38}\). Several proteins and small molecules are known to stimulate or suppress the GTPase activity associated with FtsZ polymerization\(^{29,34,41,42}\). The necessity of GTP in the cobalt hexamine trichloride induced FtsZ polymerization and the importance of GTP hydrolysis in FtsZ dynamics prompted us to examine the effect of cobalt hexamine trichloride on the GTPase activity of FtsZ. Cobalt hexamine trichloride reduced the GTPase activity of FtsZ (Fig. 4). For example, the number of moles of phosphate released per mole of FtsZ after 5 min of hydrolysis reaction in the absence and presence of 0.1 mM cobalt hexamine trichloride were determined to be 15.5 ± 1.4 and 6.4 ± 2.3, respectively (Fig. 4). The suppression of the GTPase activity of FtsZ by cobalt hexamine trichloride could be attributed to enhanced bundling and stability of FtsZ polymers. Divalent calcium, ruthenium red, and glutamate are also known to reduce the GTPase activity of FtsZ\(^{29,43}\). These molecules suppressed the GTPase activity of FtsZ by forming stable FtsZ polymers.

**Cobalt hexamine trichloride stabilizes FtsZ polymers against dilution induced disassembly**

FtsZ (25 µM) was polymerized for 30 min at 37 °C. FtsZ polymers were diluted 20 times in warm 25 mM PIPES buffer without or with different concentrations of cobalt hexamine trichloride and the samples were incubated for 15 minutes at 37 °C. The polymers were collected by high speed centrifugation. In the absence of cobalt hexamine trichloride, only 10 ± 3 % of the total FtsZ was pelleted showing that most of the FtsZ polymers were disassembled upon dilution. The sedimented FtsZ protein concentration in the pellet increased with increasing concentration of cobalt

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**Fig. 3** — Cobalt hexamine trichloride increased the sedimentable FtsZ polymer mass. [FtsZ (6 µM) was polymerized in PM buffer and 1 mM GTP at 37 °C in the absence and presence of 10, 20, 50, 100 and 200 µM cobalt hexamine trichloride. The polymers were collected by centrifugation. The polymer mass of FtsZ was calculated after subtracting the supernatant FtsZ concentration from the total FtsZ concentration].

**Fig. 4** — Cobalt hexamine trichloride suppressed the GTPase activity of FtsZ polymers. [FtsZ (6 µM) was polymerized in PM buffer and 1 mM GTP at 37 °C in the absence (●) and presence of 0.1 mM (▲), 0.5 mM (△) and 1 mM (■) of cobalt hexamine trichloride].
hexamine trichloride (Fig. 5a and b). In the presence of 0.1, 0.5 and 1 mM of cobalt hexamine trichloride, 26 ± 1 %, 54 ± 5 % and 59 ± 8 % of the total FtsZ was pelleted (Fig. 5b), indicating that cobalt hexamine trichloride stabilizes FtsZ polymers against dilution-induced disassembly. Previously, ruthenium red, divalent calcium ion and DAPI were found to prevent the disassembly of FtsZ polymers\textsuperscript{43,44}. These agents were thought to promote FtsZ assembly and to stabilize FtsZ polymers by increasing longitudinal and lateral interactions among FtsZ monomers in the protofilaments.

**Cobalt hexamine trichloride induced toroidal structures of FtsZ polymers**

In the absence of cobalt hexamine trichloride, FtsZ formed rod shaped polymers (Fig. 6a). Cobalt hexamine trichloride condensed the FtsZ polymers into ring or toroid kind of structures (Fig. 6(b-d)). In the presence of 30 \(\mu\)M of cobalt hexamine trichloride, FtsZ formed both toroidal and rod shape structures (Fig. 6b). With increasing concentrations of cobalt hexamine trichloride, the toroid forms dominated the rod structures (Fig. 6 (b-d)). The number of toroidal structures increased with increasing concentrations of cobalt hexamine trichloride. However, in the presence of 1 mM cobalt hexamine trichloride, toroid structures were not visible (Fig. 6e). As observed in the electron micrographs, the toroids became more and more numerous with increasing content of cobalt hexamine trichloride. In the presence of high concentrations of cobalt hexamine trichloride, the toroids show a tendency to be drawn together forming huge globular structures (Fig. 6e). The results are similar to our previous finding that low concentration of DEAE-dextran induced formation of ring-like polymers\textsuperscript{10}. In contrast, at higher concentrations of DEAE-dextran these rings were not visible albeit they formed huge globular structures\textsuperscript{10}. This shows that cobalt hexamine trichloride like DEAE-dextran forms toroidal structures of FtsZ polymers. The reason behind the modification of the local structure still remains an enigma. Previously, we have explained the condensation of FtsZ by multivalent cations using Manning’s counterion condensation theory\textsuperscript{46}. Therefore, cobalt hexamine trichloride being a polycation could have modified the electrostatic interactions between the FtsZ subunits through neutralization of their charges and thereby collapsed the FtsZ polymers into toroids. Though many other cationic compounds are well known to induce FtsZ polymerization resulting in various morphologies, however, toroidal morphology is not a common sight\textsuperscript{43,44}. Even though a multivalent cation like ruthenium red aided extensively in inducing FtsZ polymerization, toroid morphology was however not observed\textsuperscript{43}. CoCl\(_2\) and MnCl\(_2\) did not induce FtsZ polymerization as did cobalt hexamine trichloride.

Previously, Popp \textit{et al.}\textsuperscript{47} studied FtsZ condensation using different crowding agents. Although different suprastructures of FtsZ under various assembly conditions were reported, they did not find toroids in the presence of cobalt hexamine trichloride\textsuperscript{47}. The reasons for this difference could be different assembly conditions and use of very high concentrations of both FtsZ and cobalt hexamine trichloride in their study\textsuperscript{47}. The same group obtained toroids using additives like methyl cellulose and polyvinyl alcohol\textsuperscript{47}. Methyl cellulose and zinc chloride were found to condense \textit{Mycobacterium tuberculosis} FtsZ into toroids\textsuperscript{48}. Interestingly, zinc chloride was shown to induce
toroids in *M. tuberculosis* FtsZ at pH 7.7 but not at pH 6.6 (ref. 48). The assembly and bundling of *E.coli* FtsZ polymers were shown to decrease with increasing pH. However, toroidal assembly of FtsZ was not observed under different pHs49. These findings indicated that FtsZ assembly into toroid is not governed by a single factor, but rather by several factors. Understanding the mechanistic basis of FtsZ to form toroids will be interesting because Z-rings are torodial like structures. Furthermore, these toroids may have uses in nanotechnology. For example, these toroids may be used as templates to build toroidal resonators for electrical and thermal applications. The optical and magnetic properties of the templated materials could also be interesting.

**Conclusions**

We have studied the influence of cobalt hexamine trichloride on the modulation of FtsZ assembly. Cobalt hexamine trichloride was found to promote the
assembly and bundling of FtsZ in the presence of GTP. It promoted the stability of FtsZ polymers in association with the reduction of GTPase activity of FtsZ. More importantly, it aided the rod shaped FtsZ polymers to condense into toroids. The toroidal morphology could be adjusted by varying the concentrations of cobalt hexamine trichloride. This controlled toroidal assembly may be an advantage in regulating nanoscale systems.

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References