Chapter 2
Why Cytoskeletal Gel?

2.1 Self-Assembly and Dynamic Structure of Cytoskeletal Filaments

The cell which is surrounded by the hydrogels of ECM is also a hydrogel of cytoskeletal proteins. In vivo, cytoskeletons contribute to organization of cellular structures with the robust and dynamic nature. By the thickness of the cytoskeletal filaments, they are usually categorized into three types as microtubule (MT), actin, and intermediate filaments (IFs). In contrast to MT and actin in which amino acid sequences are relatively mutual among different species of living organisms, intermediate filaments have wider variety in the classified types. The roles of each cytoskeletal protein in relation to other cellular proteins have been intensively elucidated by biology, though it is still on the way to comprehensive understanding. Besides, physical property of the cytoskeleton has been investigated with biophysical interest. Rheometric analysis of cytoskeletal proteins as suspensions revealed the difference of viscoelastic properties among MT, actin, and IF even at a macroscopic scale [1]. This result indicates that nanometric features of unit molecules can be reflected to macroscopic ones of their suspension, presumably due to their hierarchical structure. Moreover, the hierarchical assemblies of these proteins are formed and maintained via dynamic self-assembly process. Since these filaments and the networked structures of them are physically robust with spatiotemporal organization, they are responsible not only to maintain the shapes of local cell structure or of whole cell but to give the change of these shapes and the motion with integration and synchronization upon the environmental changes. Such a property is quite unique to biological systems, when we look at them from a viewpoint of materials science. Utilization of cytoskeletal proteins for hydrogel materials, as an initial attempt, is promising to realize a new functional material with a concept of hierarchical structure. Before reviewing examples of hydrogels made up from the cytoskeletal proteins, basics about each cytoskeletal proteins, i.e., MT, actin, and IF, will be introduced below.
2.1.1 Actin

Usually, actin is the most abundant protein in eukaryotes and is responsible for maintaining the integrity and motility of eukaryotic cells [2]. Filamentous actin (F-actin, actin filament) is also known as microfilament in cells [2]. Monomeric form of actins is called as globular actin (G-actin). The G-actin consists of 375 amino acids, and its molecular weight is about 42,000 [3]. The amino acid sequences of actin are well conserved among eukaryote [3]. The G-acts can form filaments head-to-tail manner as for tubulin [2]. F-acts have a distinct structural polarity, and they consist of parallel two-stranded right-handed helical protofilaments that twist around each other [4, 5]. The structural dimension of F-actin is 6 nm in diameter [4], a persistence length of ~10 μm [6], and 72 nm in a helical periodicity [4].

Because of a structure polarity, the rate constants for association and dissociation in actin filaments at one end are much greater than the other end [2]. That is, the elongation takes place preferentially at one end of the actin filaments, and shortening occurs preferentially at the other in an equilibrium state. The faster-growing end is called the plus end or barbed end, while the slower-growing end is called as the minus end or pointed end [3]. The barbed end and the pointed end were named after the rigor state myosin-bound images of electron microscopy. The critical concentration of polymerization at the plus end is always lower than that of the minus end, and when the concentration of G-actin is between the critical concentration of a plus end and minus end, a plus end grows by polymerization, while a minus end shrinks by depolymerization [7, 8]. When F-actin reaches a stationary state (not equilibrium), both polymerization rate and depolymerization rate become equal to each other. Consequently, the length of an F-actin will not change when in a stationary state, but the actin monomers move like the athletic treadmill (Fig. 2.1). So we called this phenomenon “treadmilling” [2]. Locomotion by actin treadmilling is not limited in vitro, but is seen occurring in cellular motile events such as lamellipodia and filopodia [2]. Most cellular motile events by actin treadmilling are alternation of cell morphology and cell movement [2]. Studies of these cellular motile events are well studied and have revealed that many actin-associated proteins are required as well [9], especially actin depolymerization proteins such as ADF/cofilin which promote severing of F-actin and accelerate G-actin dissociation, promoting treadmilling [10, 11].

2.1.2 Microtubule

MT has hollow cylindrical structure which is made up from tubulin. Tubulin, that is generally called so, is a heterodimer formed of two globular protein subunits called α-tubulin and β-tubulin. Both of α- and β-tubulin subunits have a molecular weight of ca. 50,000 that is a chain of approximately 460 amino acids [12]. As 40 % of the sequences of α- and β-tubulin subunits are identical, the structures are
basically similar [13]. Remarkable difference of them is guanosine triphosphate (GTP) binding site. A β-tubulin subunit has an exchangeable GTP binding site, meanwhile that of an α-tubulin subunit is not exchangeable as it is buried between α- and β-tubulin subunits in the stable heterodimer [14]. Since GTP-bound tubulin heterodimer at the exchangeable site (GTP-tubulin) takes a conformation that is preferable for polymerization, they form MTs spontaneously; here “polymerization” means association of monomeric proteins via specific and reversible interactions not like covalent bond [2, 6]. Tandemly aligned tubulins form a protofilament, and a sheet of laterally linked protofilament becomes closed to a tubular shape in an assembling process (Fig. 2.2). An MT of typical structure has 13 parallel protofilaments with resulting geometry of 25 nm in diameter and a length that runs up to several tens of micrometers [12].

Due to the tubular shape, MT is stiffer than other cytoskeletal filaments, as the Yang’s modulus and the flexural rigidity were reported to be 1.2 GPa and $2.2 \times 10^{-23}$ N m², respectively; tubular shape has higher area moment of inertia [6, 15]. According to the direction of the uniformly arranged tubulins, MT has a polarity recognized by the faster-growing “plus end” faced with β-tubulin and the opposite “minus end” with α-tubulin. In cells, MTs are aligned in highly ordered radial structure serving as rigid skeleton, also giving the polarity. Moreover, the structure formed by assembly of MTs becomes even more complicated during cell division, where chromosomes were properly separated to daughter cells via spindle structure of MTs. In vitro, MT can be formed by inducing the self-assembly of tubulins at warm temperatures, such as 37 °C, in a buffer containing GTP and disassembles reversibly by reducing the temperature. The dynamic equilibrium
Fig. 2.2 Schematic illustration of polymerization of tubulin and the MT depolymerization. Polymerization of tubulins to MTs takes place at appropriate condition such as tubulin concentration, pH, and temperature. GTP-tubulins join tandemly to form straight linear oligomer that is called protofilament (PF). Following to this, a sheet of the PFs are formed by their lateral interactions, and this sheet will round and zip up to give tubular shape. MT has dimensions of 25 nm in diameter and the length reach to several tens micrometers in a typical in vitro preparation condition. In a depolymerization process, the tubular shape will dissociate to curve PFs due to the conformational preference of GDP-bound tubulins that is produced by hydrolyzing GTP in the tubular shape. These PFs will further disassemble to tubulins later on. In typical in vitro condition, polymerization and depolymerization can be induced by warming up to 37 °C and cooling down on ice, respectively, in a GTP abundant condition.

between MT polymerization and depolymerization has been well studied [16–19]. For instance, depolymerization of MT can be prevented by a drug called paclitaxel which is also known as an anticancer drug to disturb the MT dynamics by suppressing depolymerization. MTs in vivo are accompanied with microtubule associating proteins (MAPs), and the dynamics is controlled for the functionality.

With the polarity, MT also works as a rail that guides the MT-related motor proteins. For example, kinesin is one of the motor proteins that walk along the MT toward the plus end being energized by adenosine triphosphate (ATP) hydrolysis. Collaborating with the motor proteins such as kinesin and dynein, MTs facilitate the intracellular transport and deformation of eukaryotic cells [6, 20]. In addition to the radially organized spindles, MT also works as a skeleton of eukaryotic flagella. There, the rigidity of the MT is harnessed to orchestrate the driving forces of the interacting dynein motors by a load-dependent detachment, which realizes the macroscopic propelling motions [21, 22]. Since polarity of the MT is reflected in the
moving direction of motors, MT can be conveyed along the polarity and is coupled with ATP hydrolysis on a kinesin-immobilized surface in vitro.

### 2.1.3 Tropomyosin

Tropomyosin is categorized as cytoskeletal protein [23]. Tropomyosin is also one of actin-binding protein, which lies on the groove of F-actin and spans from five to seven actin monomers [24, 25]. Structure dimensions of tropomyosin are typically 40 nm in length and 2 nm in diameter [26]. The persistence length of tropomyosin is calculated to be 50–200 nm, meaning that tropomyosin is considered as rigid body [3, 27]. The Young’s modulus is also estimated as ~2 GPa [3], which is almost equivalent to polypropylene [28].

The rigid-body-like tropomyosin consists of a two-stranded parallel α-helical coiled-coil structure. Crick discovered that two-stranded parallel α-helical coiled-coil structure would be stabilized nonpolar amino acid residues in the intermolecular interface and outside of the interface region by polar or charged residues that interact with the partner chain and/or other proteins [29]. Therefore, a characteristic repeat of nonpolar and polar or charged residues is required. Each period corresponds almost two turns of an α-helices with 3.5 residues per turn [29]. Consequently, the repeat unit becomes seven amino acid residues [30]. In this heptapeptide repeat, each position is designed as a–g, in which positions a and d are occupied by hydrophobic residues, whereas b, c, e, f, and g are occupied by polar or charged residues [31–33]. The hydrophobic intermolecular interactions take place between residues a and d, and electrostatic intermolecular attraction takes place between e and g [32].

Amino acid sequence analysis of tropomyosins revealed that tropomyosin had the sequence characteristic to the α-helical coiled-coil protein. This analysis also showed that there is indeed a regular pattern of hydrophobic residue which extends the entire molecule (Fig. 2.3).

![Fig. 2.3](image-url) (a) 3D structure of tropomyosin (from PDB 1C1G). (b) The unit of heptapeptide repeat in the amino acid sequence of tropomyosins. Seven positions are described as a–g. Adapted with permission from Langmuir 31, 2826–2832. ©2015 American Chemical Society.
2.2 Multi-Scale Hierarchy: Origin of Emergence

As described in the preceding chapter, biopolymers are especially characterized by their ability to form higher-ordered network structure through self-organization without denaturation, i.e., keeping their second- and third-ordered structure of the components, they form aggregated fourth-order and even network structures. That is nature’s preferred way of building their body with multi-scale hierarchical and functional structures on various scales.

An important fact is that the emergent function is often arisen by the newly organized macroscopic structure which formed in an environment as a result of a very large nonlinear ensemble of the interactions at a lower hierarchical or constituent level. However, it should be emphasized although the highly hierarchical structure is the origin to display the emergence, it does not always induce emergence since a large number of interactions sometimes work against the emergence by interrupting the constitutional element and by producing noise without any periodic ordering. Thus, the organization with hierarchical structure is not every time the essential factor of emergent function. Then, what is the requirement to arise the emergence?

Let the term “emergence” in this case be defined as functions of integration and synchronization expressed at higher hierarchical level. The reason is these would be one of the central functions and natures characterizing the living organism, and through it one may lead to solve the mechanism of emergent function.

We can see suitable example of emergence in the process of muscle contraction of mammals through multi-scale hierarchy, i.e., in the cooperative and synchronized sliding displacement of actin–myosin arrays in the sarcomere. In fact, elucidation of these motor protein mechanisms at single molecular level has recently been progressed to a great extent especially concerning several popular motor proteins such as F$_1$F$_0$/ATPase, actin–myosin, and MT–kinesin systems [1, 34, 35]. This exploration on the motor protein mechanism has been extended from single molecule to multi molecular assembly as several cases have been reported [15, 36, 37]. Thus, it was found that the displacement of actin–myosin is 5–8 nm and the response time is ~5 s. The contractile force is in order of several pico-Newton on the molecular level. As well known, actin and myosin in muscle are assembled into filaments to form parallel lattice called sarcomere, and sarcomeres are further piled up in a hierarchical manner. The contraction process with these characteristics at the actin–myosin level is integrated across a multi-scale hierarchies, i.e., from the actin–myosin level via protein thread, to the sarcomere, myofibrils, skinned fiber, muscle fiber, and finally muscle level on an individual. Through this the muscle can exert several centimeters of displacement, hundred Newtons of contractile force with 40 ms of response time, and the degree of integration of these are such large as $10^8$, $10^{14}$, and $10^{-2}$, respectively, and they are not displayed unless synchronization system does not work. This is one of typical “emergence” of actin–myosin interaction-triggered molecular deformation, and this was integrated to a macroscopic change through the hierarchical structure with a spatial-temporal regulation.
How is the integration performed across the hierarchy and what is the responsible structure of biomolecules to the cascade process? That is the reason why we need to build up the integrated hierarchical 3D-structured motor protein systems.

### 2.3 Cytoskeletal Protein Gels: Multi-Scale Hierarchical Supra-Macromolecular Gel (MHSMG)

As will be described in Sect. 2.3.1 in detail, cytoskeleton is the filaments system composed of three types of self-assembled cytoskeletal protein filaments—actin, tubulin, and intermediate. Since these filaments are all physically robust with spatially self-organized bundle structure, they are able not only to maintain the shape of the cell but to give the motion upon changing the environment. There, the formation of highly hierarchical bundle structure is crucial for their functions.

Self-organization is the process by which isolated components organize autonomously and spontaneously into the ordered structure. Here, two types of self-organization processes could be considered: one is passive and the other is active self-organization. The distinction between these two refers to the thermodynamic description of the resulting assemblies—the former is being equilibrium structures and the latter is a stable non-equilibrium structure maintained at a steady state by a constant supply of energy ($E$), which is subsequently dissipated via the entropy-producing process associated with the interactions of the system’s components. In some innovative research works, these cytoskeletal proteins have been integrated toward the hierarchical complex level through various self-organization processes, keeping their basic properties unaltered. However, the great difficulty lies in how to integrate them to form robust three-dimensional gel with network structure keeping the functions as nature have.

This would not only lead to discover various features correlating to govern the emergent function but expand the utility of integrated artificial bio-machine over the nano-device addressed by using motor proteins in a single molecular level (Fig. 2.4).

#### 2.3.1 Actin Gel

In vivo the mechanical properties of actin cytoskeleton are regulated predominantly by actin-binding proteins which enable to align the filament, form bundles, cross-link the filament, and, consequently, form mesh-like actin hydrogels, branched dendritic networks or bundles [2]. Due to the functions of the actin-binding protein, cytoplasmic actin hydrogels obtain the storage modulus $G'$ of order $10^{-1}$–$10^2$ Pa, the value which is required to maintain the shape of the cell. Expecting to obtain the similar actin gel organized in vitro, we attempted to form chemically cross-linked actin gel by using tetra-PEG-maleimide which behaves as a cross-linker of actin
fibers [38, 39]. The $G'$ of such chemically cross-linked actin gel measured by mechanical oscillating strain showed as high as $10^3$ Pa which substantially exceeded those of cytoplasmic actin gels formed by actin-binding proteins [40]. Our PEG-cross-linked gel also exceeded 2–3 orders of magnitude mechanical performance comparing with entangled F-actin filaments. These results indicate that the chemical cross-linking of F-actin fibers can supply robust and mechanically stable hydrogel. It was also found that $G'$ of chemically cross-linked actin gel exceeded that of synthetic polymer gels with flexible chain network as polyacrylamide gel under the same network density. This is apparently attributed to very rigid and coiled-coil filamentous nature of the network composed of G-actin bundles with high-ordered structure dimension.

Particular interest is the fact that PEG-cross-linked F-actin gel undergoes reversible sol–gel transition in vitro by changing the ionic concentration (Chap. 3). While the salt concentration is high, the cross-linked F-actin keeps its gel state, but it depolymerizes to give G-actin and transfers to the sol state, changing $G'$ values in three orders of magnitude from $10^3$ to 100 Pa. The F-actin gel also undergoes the sol–gel transition by applying a relatively small amount of oscillatory share strain (20 %, 1 Hz) and exhibits self-repairing ability. When the strain is removed, the depolymerized G-actin immediately and spontaneously forms F-actin gel with the higher-ordered structure and recovers the original $G'$ value. The velocity of F-actin
network formation of the decomposed G-actin is much higher than that of native G-actin. This indicates that the polymerization and depolymerization processes are highly cooperative.

2.3.2 Microtubule Gel

In the similar manner, chemically cross-linked tubulin dimer undergoes polymerization to form MT (microtubule) hydrogel (cross-linked by tetra-PEG-tubulin conjugate) by increasing temperature from 4 °C. While temperature is below 25 °C, $G'$ of the solution of tubulin dimer and tetra-PEG conjugate mixture does not increase, showing an order of $10^{-1}$ Pa, but it abruptly jumps up showing $G'$ of $10^3$ Pa at 26 °C and indicates chemically cross-linked MT gel has been organized [40]. This process is reversible and the depolymerization of cross-linked MT hydrogel occurs to give tubulin dimer by lowering temperature. Thus, the MT hydrogel varies its $G'$ values as much as three orders of magnitude (sol–gel transition) by temperature change.

The transition state of MT formation (defined as the temperature at which half-log $G'$ shows) was 26 °C for the case of MT hydrogel formation, and it was 30 °C for uncross-linked MT formation (Chap. 4). The decreased transition temperature of the cross-linked MT gel indicates that the formation of cross-linked MT is thermodynamically favorable than that of uncross-linked MT formation. In addition, the rate of formation of cross-linked MT hydrogel from depolymerizes tubulin (the slope of increase in $G'$ both at the beginning and at the transition state) was much larger than that of uncross-linked MT was much higher than the native tubulin. All these experimental facts show the enhanced cooperativity of the polymerization process of the cross-linked MT presumably due to increased molecular mass and also decreased mobility of growing cylindrical MT network.

Apart from the typical synthetic polymer gels, the network of cytoskeletal hydrogels has three intrinsic characteristics: Firstly, their molecular mass: Synthetic polymer gels are made of polymer networks consisting of low molecular compounds (monomers), while the cytoskeletal hydrogels consist of the network of self-organized assembly of the globular protein—G-actin or tubulin— which is biopolymer of molecular weight of more than $10^4$ Da. The latter makes supra-macromolecular mass, attaining more than 10. Secondly, their rigidity: The network of synthetic polymer gels is composed of flexible polymer chains which sensitively changes its conformation upon changing the environment and exhibits the swelling and contracting phenomenon, thus, usually amorphous. On the other hand, the network of the cytoskeletal hydrogels consist of the coiled-coil (actin) fiber or cylindrical tube composed of 13 protofilament units array, both brings about extremely rigid and robust properties and therefore gives the highly organized structure to the hydrogels. Finally, the network of synthetic polymer gels is formed of covalently bound which does not undergo polymerization and depolymerization reversibly to give sol–gel transition as cytoskeletal hydrogels.
We consider that high-ordered structural hierarchy is critical to exert these enhanced cooperativity and synchronization induced by chemical, mechanical, and thermal stresses. The network of these cytoskeletal hydrogels consists of sterically well-defined geometrical structure composed of globular proteins with tertiary-ordered structure, and the network fiber itself is made of F-actin fiber and MT which have fourth-dimensional order. Therefore, the described PEG-cross-linked cytoskeletal hydrogels could be categorized as multi-scale hierarchical supramacromolecular gel (MHSMG) with a fifth-dimensional-ordered structure, and due to this, the gel could exhibit described emergence functions beyond the hierarchies.

2.4 Bio-Motor Gel with Emergent Function

Among cytoskeletal proteins, actin–myosin and microtubule–kinesin are the constituent components of biological power systems as well as smallest machines that can provide mechanical work. Motor proteins can be categorized into two groups as rotating motors and linear motors: linear motor proteins as actin–myosin and MT–kinesin systems which are found in relatively larger living organisms and organs of animals, while rotating motors are observed in bacterial flagellum and mitochondrial. The former have been widely studied [41].

Since biological motors driven by the conversion of chemical energy to mechanical energy are much more efficient than the man-made machines and they possess of immense potential in biotechnology and biomaterials science, a variety of ATP-fueled bio-actuators using actin–myosin and microtubule–kinesin actin–myosin as building block have been introduced, although most of them are performed mainly on two-dimensional experimental conditions [6, 12, 16, 17, 21, 22, 42–44].

Associating with emergent functions in living system, the study of complex hierarchical three-dimensional structures of constituents and such multifunctional complex 3D structures has long been made based on self-organization principle. It was found that chemically cross-linked electrostatically complexed actin gel with synthetic polycations, several tens of times the length of native actin filaments (F-actin) move along a chemically cross-linked myosin fibrous gel (1 cm long and 50 mm in width) by coupling to ATP hydrolysis (nano-bio-machine) [45, 46]. The muscle proteins could be successfully tailored into desired size and shape by controlling factors such as time and protein concentration and orientation direction of actin fibers without sacrificing their bioactivities.

The mean velocity on the non-oriented myosin gel was 0.69 μm/s, while that on the oriented myosin gel was 0.83 μm/s and much exceeded the velocity of native F-actin. The velocity of the motion of F-actin gel is dominated by the polarity of F-actin of the complexes which was, in turn, determined by the chemical structure of polymeric cations: Polycations carrying charges in the side group, produced F-actin complexes with high polarity, and those having charges on the
chain backbone produced the complexes with lower polarity. Thus, despite its increased mass (several tens to hundreds of time the volume of the native F-actin) and decreased effective surface for ATP hydrolysis, the actin gels move on the covalently cross-linked myosin gel with an increased velocity. This is rather surprising since the interaction between the myosin gel and the actin gel can occur only at the two-dimensional interface, and due to cross-linking, a considerable number of actin and myosin molecules are not involved in the sliding motion. In addition the F-actin bundles exhibited a synchronized motion with periodical oscillating waves as if it is one individual worm. This means that the self-assembled and covalently bound actin gel and myosin gel exert a highly cooperative synchronized motility coupling to ATP hydrolysis.

As well established, the motion of F-actin is performed by the specific coupling with myosin under the action of ATP, and accordingly, the interaction between F-actin and myosin on the molecular level is critical. In the case of cross-linked F-actin and myosin gels, the real surface on which the motion of F-actin is performed would certainly be restricted due to their three-dimensional structure. Nevertheless, they could exhibit motility as high as their constituents: F-actin and myosin. These could be classified as examples of “emergent” functions which arose through highly hierarchical structure of F-actin and myosin three-dimensional gels. Thus, the “motility” which is one of critical and essential function of living organism could successfully be associated with rather simple “chemical” structure of the synthetic macromolecules.

We have also demonstrated that the chemically cross-linked filamentous microtubules (MT) network exhibits an enhanced and prolonged motility on the kinesin-immobilized surface comparing with that of uncross-linked MT, although the MTs were higher-ordered molecular structure, but randomly cross-linked and no specific control of polarity of the network was made [15]. Thus, the MT network successfully conveyed the silica microbeads placed on it at the peak rate of 1.5 μm/s or for several tens of micrometers in distance for 1000 s. The reason of the enhanced velocity of the MT network was associated with the elastic force generated and stored in the MT network which, in turn, temporally and spatially releases to accelerate the beads on the MT network (mechanochemical effect) where the synchronized mechanochemical response is essential [47]. In both cases, the proteins are characterized by their ability to form the higher-ordered molecular structure to form three-dimensional gels through self-organization without denaturation, i.e., keeping their third-order and even aggregated fourth-order structures.

Thus, the “motility” which is one of the critical and essential functions of living organism could successfully be associated with rather simple “chemical” procedure of the motor proteins to give multi-scale hierarchical supra-macromolecular gels (MHSMG) with motility functions [15]. The point is these artificial bio-machines can move much increased velocity and much increased power (energy) than those of the native proteins. This means the covalently bound protein gels are able to exert emergent function cooperatively synchronizing and integrating between component network fibers.
References

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