1 Polysaccharide Carriers for Induction and Evaluation of Tissue Regeneration and Drug Delivery

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1.1 Introduction

Induction of tissue regeneration has been highly expected as a new field of medical treatment covering or compensating two advanced medical therapies; reconstruction and organ transplantation therapies. The basic idea of tissue regeneration induction is to regenerate or repair the injured or lost tissues and substitute organ functions based on the natural self-healing potential of patients themselves. With the recent rapid development of cell biology, it has been possible to make use of various progenitor and stem cells with high potentials of proliferation and differentiation for cell-based induction of tissue regeneration. It has been demonstrated that cells themselves have good therapeutic potentials in terms of their inherent targetability to the site injured or biological properties. However, the therapeutic efficacy of cells transplanted is not always as high as expected, which is one of the largest problems in cell therapy. This is because the survival rate of cells transplanted is low, and consequently the biological functions of cells are not always expected in the body. To tackle the problem, it is indispensable to develop materials, technologies, and methodologies to provide the cells a local environment where the survival and biological functions of cells transplanted can be maintained or enhanced. On the other hand, the technologies and methodologies to evaluate the extent and process of tissue regeneration still depend on the conventional diagnostic methods such as histological, biochemical, and morphological examinations. Clinical availability of these examinations is limited in terms of their invasiveness and

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reliability. Therefore, it is also necessary to develop new systems for the non-invasive evaluation of tissue regeneration. On the basis of these requirements, many types of biomaterials (polymers, metals, ceramics, and their composites) as cell scaffolds and drug delivery carriers have been designed and created to achieve the effective induction and evaluation of tissue regeneration.

Polysaccharide is a monosaccharide-repeated biopolymer with various glycosidic bonds and composes a part of living body such as wall of plant cells, exoskeleton of arthropods, connective tissue or cell surface as well as serves as energy yielding fuel. Most of polysaccharides have long histories of the medical, pharmaceutical, and food applications, and their biosafety and bioavailability have been proven based on the practical usage. Since polysaccharide has reactive groups, such as hydroxyl, carboxyl or amino groups, the chemical modification is easy to give it chemical, physical, and biological properties and to form several types of structures. Taken together, polysaccharide is expected to be the one of the most feasible biomaterials for the effective induction and evaluation of tissue regeneration. In this chapter, the current status in the induction and evaluation of tissue regeneration based on polysaccharide as a cell scaffold and drug delivery carrier is overviewed. Additionally, recent researches on the manipulation and tracing of stem cells and molecular imaging of tissue regeneration with a nano-sized polysaccharide construct are introduced.

1.2 Role of Biomaterials for Induction of Tissue Regeneration

It is well recognized that cells are present in the living tissue interacting with the extracellular matrix (ECM) of a natural scaffold for their proliferation, differentiation, and morphogenesis. When the body tissue is largely lost, the ECM itself also disappears. In such a case, only by supplying cells to the defect, we cannot always expect the natural induction of tissue regeneration. There are two approaches to induce the effective tissue regeneration based on the concept of tissue engineering[1], which provides cells transplanted with a suitable environment where the cells maintain their biological functions such as survival, proliferation, and differentiation (Figure 1.1). One is to provide a temporary scaffold and bioactive substance (bio-signaling protein or the related gene) to the defect for the proliferation and differentiation of cells. The other is to transplant cells with the biological function manipulated by the cellular

internalization of bioactive substance. It is indispensable to design the practical biomaterials that support these approaches. Following sections describe several key points for biomaterials design to make these approaches effective.

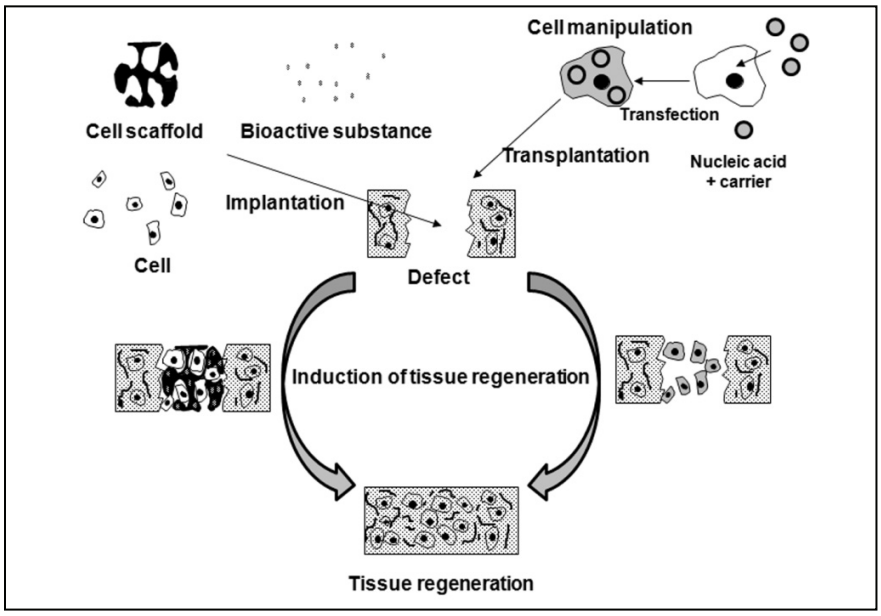


FIGURE 1.1 Tissue engineering approaches for induction of tissue regeneration.

Cell scaffold materials should be safe contacting with cells transplated and have an adequate mechanical strength during the process of tissue regeneration while they should disappear after the tissue regeneration. Therefore, the cell scaffold materials should be non-toxic, biocompatible, and biodegradable. The ECM is composed of structural proteins (collagen or elastin etc.), adhesion proteins (fibronectin, laminin, vitronectin, etc.), and several polysaccharides (glycosaminoglycans; hyaluronic acid or sulfated polysaccharides). In this context, these kinds of materials are mainly used for preparation of scaffolds to mimic a native extracellular environment. Since water-soluble polysaccharides are rapidly diffused to disappear from the site placed, they should be insolubilized to use as a scaffold.

Insolubilization is achieved by

(i) The ionic interaction with low-molecular-weight counter ion,
(ii) The formation of polyion complex with polyelectrolytes having an opposite charge,

(iii) The formation of interpenetrating polymer network with nonionic polymer,

(iv) The acquisition of temperature sensitivity by modification with a hydrophilic or hydrophobic residue, and

(v) The formation of chemical bonding by a crosslinking reagent or radiation irradiation.

Three-dimensional and sponge scaffolds with a pore interconnectivity should be prepared to provide cells with the sufficient surface area for their initial attachment and the adequate supply of nutrient and metabolite. Processing methods to provide the porous structure in the scaffolds include particle reaching, freeze drying, phase separation, fiber meshes, fiber bonding, melt processing, batch forming, electrospinning, and rapid prototyping[2]. Different shapes of three-dimensional porous scaffolds, such as granule, sheet, fiber, fiber mesh, and non-woven fabric, can be obtained by various processing methods. In addition to the porous scaffold, the preparation of injectable scaffolds is another feasible approach for the induction of tissue regeneration. The advantage of injectable scaffolds is to allow to avoid the invasive surgical implantation and to easily fill the individual and irregular shape of defect. Injectable scaffolds can be divided into two types; microparticle and *in situ* forming scaffolds. Microparticle scaffolds are prepared by the methods of precipitation, simple or complex coacervation, spray drying or suspension, emulsion, and dispersion polymerization/crosslinking. *In situ* forming scaffolds are prepared via thermoplastic pastes, *in situ* polymerizing/crosslinking, *in situ* precipitation, and gelation under an environment-based stimulation. Cell scaffolds can be prepared from not only one kind of biomaterial but also a biomaterial-biomaterial composite. By making use of the composite, cell scaffolds with an easy processing of preparation or an improved biological or mechanical property can be obtained.

Combination of scaffold with a bioactive substance efficiently induces tissue regeneration. Since the bioactive substance rapidly diffuses from the injected site and is enzymatically digested or deactivated, it is necessary to develop the technologies and methodologies to effectively deliver the bioactive substance to the site to be regenerated based on the concept of drug delivery system (DDS). DDS is a technology which allows a drug to act at the right time the right site of action at the appropriate concentration. The objectives of DDS include the controlled release, the life-time prolongation, the accelerated permeation and absorption, and the targeting of drug (Figure 1.2). Various biomaterials

have been extensively used to achieve each DDS objective. Since most of scaffold biomaterials are biodegradable, they are expected to be applied for the controlled release of bioactive substances by their incorporation into the scaffold. Incorporation of bioactive substances is carried out during or after processing of scaffold preparation, which depends on its sizes. Incorporation into a biomaterial scaffold enables the bioactive substance to protect from the enzymatic attack and gradually release in the site to be regenerated. Release profile of bioactive substance is governed not only by the degradability or structure change of scaffold biomaterials, but also by the interaction strength with the scaffold biomaterial chain. Therefore, it is necessary to design and construct scaffold biomaterial based on these aspects for the ideal controlled release of bioactive substances.

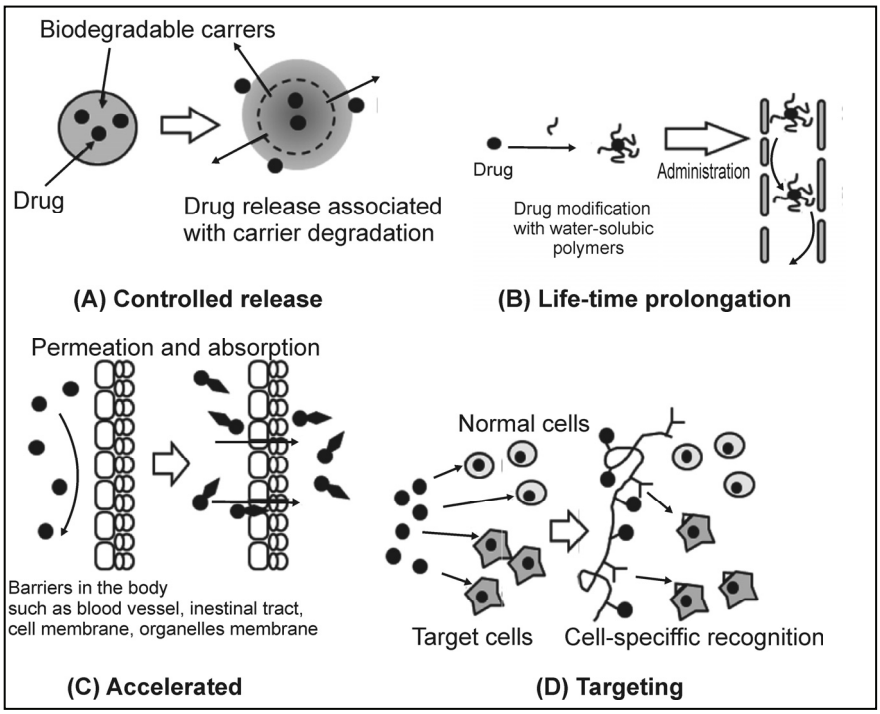


FIGURE 1.2 Objectives of DDS.

Transplantation of cells artificially manipulated to obtain biological functions, such as the survival, proliferation, differentiation, and therapeutic abilities, is expected to promote the induction of tissue regeneration compared with the original cells alone. Generally, nucleic acids, such as plasmid DNAs, antisence oligonucleotides or small

interfering RNAs (siRNA), are mainly used as bioactive substances for the manipulation of cell functions. The manipulation of cell function is fundamentally achieved by the transfection procedure of the related nucleic acids for the cells. Therefore, it is necessary for successful cell manipulation to develop materials, technologies, and methodologies for safe and efficient transfection of nucleic acid. The transfection system is generally divided into two categories in terms of carrier materials of nucleic acids: viral and non-viral systems. For the viral system, the carrier of retrovirus, lentivirus, adenovirus, and adeno-associated virus, has been used to be potentially efficient, although there are several issues to be resolved for the clinical applications, such as the antigenicity and toxicity of virus itself or the possibility of disease transfection. Therefore, efficient technologies and methodologies of transfection without using the virus are highly expected. The non-viral system has several advantages in terms of its safety and no limitation in the molecular size of nucleic acid applied. However, the low transfection efficiency is one of the major drawbacks for the research and therapeutic applications. In this circumstance, various experimental trials have been performed to develop the physicochemical and biological properties of non-viral carriers. Nucleic acid in the naked form is a polyanion of phosphate group-repeated chain and has an expanded molecular structure due to the intermolecular repulsion force of negative charge at the physiological pH. Therefore, it is well recognized that the nucleic acid cannot interact with the cell membrane negatively charged due to the electrostatic repulsion, and consequently hardly be internalized and subsequently transfected. On the basis of these findings, the non-viral carriers of cationized polymers[3-5] and cationized liposomes[6-8] have been developed to allow nucleic acid to effectively internalize into cells for transfection (Figure 1.3). The cationized materials enable the nucleic acid to form a complex with a molecular size and surface charge which are suitable for the cellular internalization and consequently enhance the complex internalization. However, this method is only to increase the non-specific cellular internalization of nucleic acid through the simple electrostatic interaction between the complex and cell surface. To enhance the specific cellular internalization of complex by making use of biological interactions, there have been several research trials with ligands specific for cell surface receptors, such as folate, transferrin, mono- or oligosaccharides, peptides, and proteins. Modification of the ligand will enable nucleic acid to internalize into cells in a cell-specific manner. It is also important for effective biological expression of nucleic acid to consider the intracellular trafficking of nucleic acid-carrier complexes or their stability in the cell.

In the case of normal intracellular trafficking, the nucleic acid-carrier complex internalized via an endocytosis pathway is carried into the endosomal compartment, followed by the lysosomal degradation. Therefore, the carrier should be molecularly designed to allow the nucleic acid-carrier complex internalized to effectively escape from the endosomal compartment into the cytoplasm one. For example, when covalently linked with a peptide capable to disrupt the lysosomal membrane under an acidic condition where lysosomal enzymes biologically function, a carrier effectively enhanced the expression level of nucleic acid[9]. It is reported that the carrier covalently linked with functional groups having a buffering capacity to accelerate the endosomal escape, so-called "proton sponge effect", enhanced the expression level of nucleic acid[10]. Furthermore, the carrier covalently linked with a peptide of nuclear localization signal (NLS) enabled a nucleic acid to positively deliver to the cellular nucleus[11]. There have been several researches to combine nucleic acid-carrier complexes with physical stimuli, such as pressure, electricity, ultrasound, magnetism, and light, to enhance or regulate the level and pattern of nucleic acid expression.

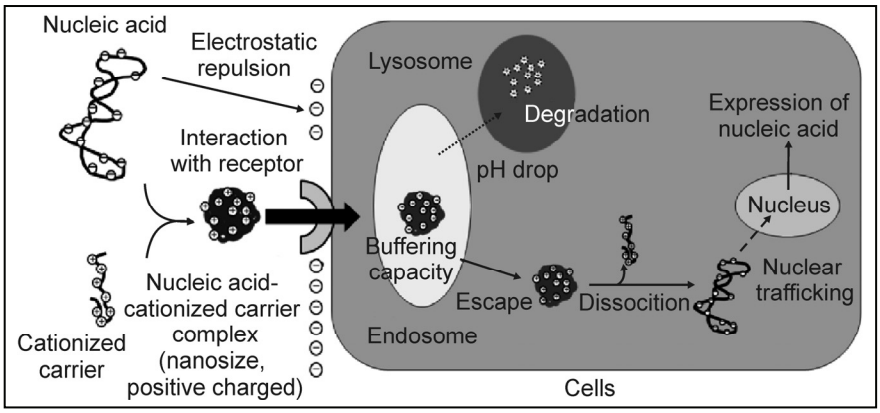


FIGURE 1.3 Internalization and intracellular trafficking of nucleic acid complexed with a non-viral cationized carrier for the expression.

1.2.1 Polysaccharide as a Cell Scaffold to Induce Tissue Regeneration

As described above, many kinds of biomaterials have been used for the preparation of scaffolds. Polysaccharide is largely non-toxic, biocompatible, biodegradable, and often degraded by specific enzymes in the body. In addition, the bioadhesive property and low cost for acquisition are advantageous over other biomaterials. It is well known

that glycosaminoglycans (GAG) are one component of ECM. The GAG possess biological characteristics of the binding and modulation of growth factors and cytokines of bioactive substances, the inhibition of proteases' function, and the involvement in adhesion, migration, proliferation, and differentiation of cells[12]. Therefore, making use of polysaccharide with chemical structures similar to that of GAG is quite advantageous to prepare biologically functional cell scaffolds. Furthermore, several polysaccharides have properties of self-insolubilization or *in situ* crosslinking, which have been practically used as injectable scaffold systems. There have been many types of polysaccharides to use as cell scaffolds to induce regeneration of various tissues. The following describes the representative polysaccharides as cell scaffold for induction of tissue regeneration.

Alginate (Figure 1.4A) is an anionized polymer linked with β-D-mannuronic acid and α-L-guluronic acid units. Alginate is insolubilized to form a hydrogel in aqueous solution by the ionic interaction of α-L-guluronic acid units with divalent cations such as calcium or barium ions. The crosslinking extent of hydrogel is changed by the composition of α-L-guluronic acid in the alginate. Since the hydogel is formed under a mild condition, alginate has been widely used as cell scaffolds for induction of various tissues regeneration. The hydrogel or beads of alginate scaffolds can be prepared by applying the alginate aqueous solution containing the desired bioactive substance or cells into a solution containing divalent cations. Other insolubilization methods to prepare the cell scaffold include chemical crosslinking[13], complex formation with cationized polymer[14], and composite formation[15]. Alginate has often been mainly applied for the induction of cartilage regeneration because the structure is similar to the GAG of chondrocytes' ECM and that the three-dimensional culture of chondrocytes is required for the induction of cartilage regeneration[16]. The cartilage regeneration is induced by alginate beads, discs, hydrogels, and sponges encapsulating chondrocytes or progenitor/stem cells combined with several bioactive substances[17-20]. Other researches on the regeneration induction of various tissues, such as vascular, peripheral nerve, intervertebral disc, liver, and pancreas, with alginate scaffold have been also reported[13,21-24].

Chitosan (Figure 1.4B) is a cationized polymer composed of N-acetyl-D-glucosamine and D-glucosamine. Chitosan is enzymatically degraded by the lysozyme. The water-solubility and biodegradability are altered by the relative proportions of N-acetyl-D-glucosamine and D-glucosamine in the chitosan molecule and the solution pH. To use as scaffolds, chitosan

is crosslinked mainly by the crosslinking reagent or formation of polyion complex. Different shapes of chitosan-based scaffold have been prepared, for example, granules, fiber, tube, sponge or microsphere. Since the structure of chitosan is also similar to the GAG of chondrocytes ECM[12], chitosan microsphere scaffold have been used for cartilage regeneration[25]. In addition, it has been reported that chitosan has a strong tissue-adhesive property and enhances blood coagulation[26]. In this context, chitosan hydrogel scaffold has been used as a wound dressing[27]. Regeneration of other tissues, such as bone, vascular, and peripheral nerve, has been reported by using the scaffold with or without the incorporation of the related bioactive substance[28-30].

Hyaluronic acid (Figure 1.4C) is an anionized polymer composed of α-1,4-D-glucuronic acid and β-1,3-N-acetyl-D-glucosamine. Since the hyaluronic acid is highly water-soluble, the chemical modifications are required to insolubilize. Hyaluronic acid modified with benzyl ester groups is called HYAFF®, which is widely used for a cell scaffold material[31]. Various shapes of HYAFF® have been produced and applied for the regeneration induction of cartilage, vascular, and adipose tissues[32-34]. Other researches have been also reported on the regeneration induction of spinal cord[35] and skin[36] tissues with hyaluronic acid-based scaffold.

In other polysaccharides, scaffolds prepared by agarose (Figure 1.4D)[37], cellulose (Figure 1.4E)[38,39], chondroitin sulfate (Figure 1.4F)[40-43], dextran (Figure 1.4G)[44,45], gellan gum (Figure 1.4H)[46] or starch (Figure 1.4I)[47,48], have been used for induction of tissue regeneration by making use of their individual properties.

1.2.2 Polysaccharide as a Transfection Carrier to Induce Tissue Regeneration

Among the non-viral materials applicable for the transfection carrier of nucleic acid, polysaccharide has several advantages over other carrier materials. Since polysaccharide has reactive groups, the chemical modification can be readily made to change the chemical, physical, and biological properties. Another noticeable feature of polysaccharide is to compose of different sugars which can be recognized by the corresponding cell receptors of sugar specificity. This biological recognition not only permits the receptor-specific targeting of agents to the cell, but also accelerates their cell internalization via the receptor-mediated endocytosis. Based on these findings, several researches for the efficient transfection of nucleic acid have been carried out with cationized polysaccharides.

Dimethylaminoethyl dextran (DEAE-dextran) is one of the oldest carriers which have been widely used for the transfection of nucleic acid into mammalian cells cultured[49]. The DEAE-dextran of positive charge can interact with the nucleic acid of negative charge and consequently form the polyion complexes of nucleic acid and DEAE-dextran. It is recognized that the nucleic acid-DEAE dextran complex is adsorbed onto the cell surface through the simple electrostatic interaction force, followed by internalizing into the cells via an endocytosis pathway. The easy procedure is a big advantage of this method, but the cytotoxicity of DEAE-dextran itself and poor reproducibility are practically problematic.

Chitosan itself is positively charged at a high density and interacted with the nucleic acid of negative charge, which is consequently widely used as a carrier of nucleic acid[50,51]. There have been many research results to investigate the effect of physicochemical properties of nucleic acid-chitosan complexes on the efficiency of gene transfection[52-54].

Sakurai and Shinkai discovered that schizophyllan (Figure 1.4J) can form a hetero-triple helix with a single chain of nucleic acid[55] and is used as a carrier of oligonucleotides[56]. An oligoamine-introduced schizophyllan strongly interacted with nucleic acid to improve the stability and schizophyllan covalently linked with octa-arginine (R8) and arginine-glycine-aspartic acid tripeptide (RGD) enhanced the biological effect of antisense oligonucleotide[57].

For polyion complexation with nucleic acid, various cationized dextrans with different molecular weights and cationized extents were prepared by the reductive amination of oxidized dextran. The physicochemical properties of nucleic acid-cationized dextran complexes to enhance the expression level of nucleic acid were systematically optimized[58,59]. A cationized dextran modified with the oleate residue of hydrophobicity enabled nucleic acid to increase the affinity for the cell membrane and consequently enhance the expression of nucleic acid[60].

Cationized polymers as a carrier of nucleic acid modified with a side chain of a polysaccharide have been also reported. Dextran with a molecular weight of 1,500 was modified to polyethyleneimine (PEI). The dextran-grafted PEI with an optimal extent of dextran grafted not only reduced the cytotoxicity of PEI itself, but also enhanced the expression level of nucleic acid to a higher extent than that of original PEI[61]. A poly-L-lysine grafted by hyaluronic acid with a molecular weight of 15,000 enabled a plasmid DNA to target to the sinusoidal endothelial cells expressing a hyaluronic acid receptor[62].

Since stem cells possess their inherent high potentials of proliferation and differentiation into different cell lineages, they have been widely investigated for their clinical applications to induction of tissue regeneration[63]. Since transfection of nucleic acid can manipulate stem cells in terms of the biological functions as well as the proliferation and differentiation abilities, it is necessary to develop the efficient transfection technologies of nucleic acid usable as a tool for the basic research of stem cells biology and medicine. For example, induced pluripotent stem (iPS) cells were prepared by the transfection of nucleic acids for terminally differentiated cells[64]. In addition, stem cells manipulated by nucleic acids to activate and improve the biological functions can be used for cell transplantation therapy. Here, recent research results about the non-viral carrier of cationized polysaccharide for stem cells aiming at the enhancement of induction of tissue regeneration are introduced.

We have explored a cell-specific gene carrier of polysaccharides which can be recognized by the cell surface receptors of sugar-recognition for enhanced expression of nucleic acid. Pullulan (Figure 1.4K), dextran, and mannan (Figure 1.4L) were used as the starting polysaccharides of transfection carrier[65]. To cationize the polysaccharide for formation of polyion complex with plasmid DNA, the spermine of a polyamine present in the body was introduced to the hydroxyl groups of polysaccharide by a carboxydiimidazole activation method. Complexation with the polysaccharide derivatives enabled a plasmid DNA to enhance the expression level of mesenchymal stem cells (MSC) to a significantly high extent compared with that of LipofectAmine 2000® commercially available, while the enhanced gene expression depended on the polysaccharide type (Figure 1.5). The complex of spermine-pullulan and plasmid DNA showed the highest level of gene expression among the plasmid DNA complexes with other cationized polysaccharides. An inhibition assay with asialofetuin which is a ligand of the sugar-recognizable cell surface receptors revealed that the blockage of cell receptors suppressed the level of gene expression. These findings suggest the possibility that the plasmid DNA complex with the cationized polysaccharide derivative is selectively internalized into cells through a sugar-specific receptor of cell surface, resulting in the enhanced gene expression. The similar enhancement of gene expression by the cationized polysaccharide was observed for other cells, such as embryonic stem cells and adipo-derived stromal cells (unpublished data).

MSC are being expected as one of cell sources usable for cardiac reconstruction because of their differentiation potential and ability to

supply growth factors. However, the therapeutic potential of MSC is often hindered by the poor viability at the transplanted site. Therefore, as one trial to overcome this issue, a non-viral carrier of cationized polysaccharide is introduced to manipulate MSC for activation of the biological functions[66]. When manipulated by the spermine-dextran complex with plasmid DNA of adrenomedullin (AM), MSC secreted a large amount of AM which is an anti-apoptotic and angiogenic peptide (Figures 1.6A and B). Transplantation of AM-manipulated MSC (AM-MSC) significantly improved cardiac functions after myocardial infarction compared with that of MSC alone (Figure 1.6 C).

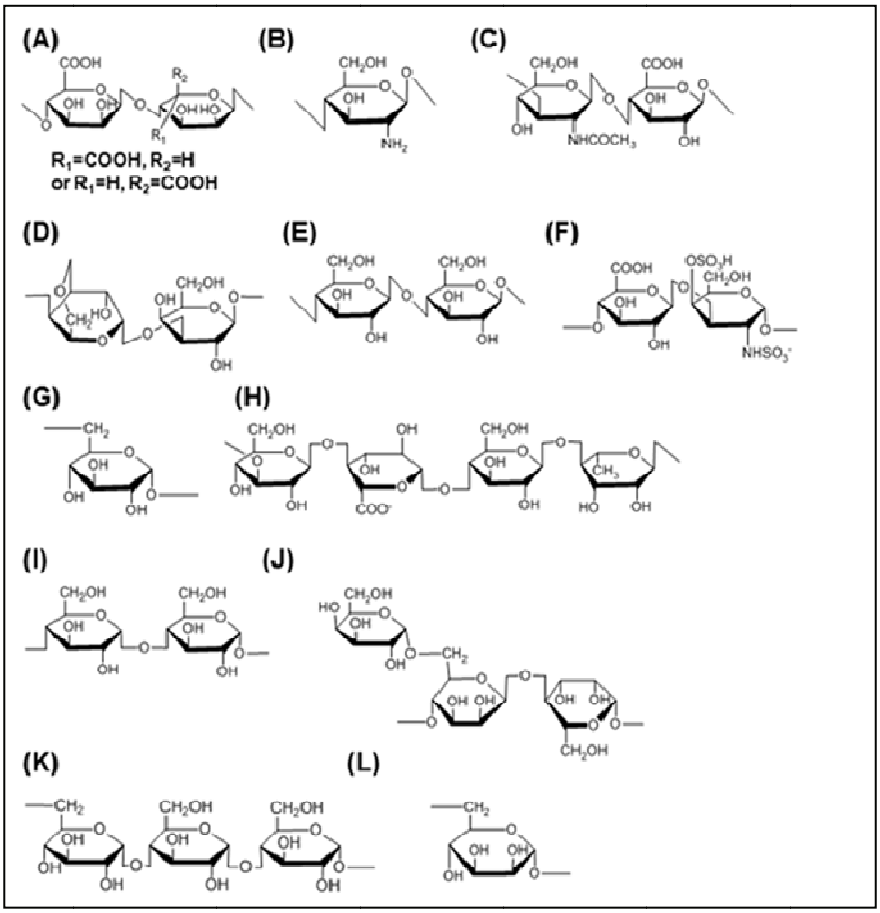


FIGURE 1.4 Chemical structures of polysaccharides used for cell scaffold and drug delivery carrier. (A) alginate, (B) chitosan, (C) hyaluronic acid, (D) agarose, (E) cellulose, (F) chondroitin sulfate, (G) dextran, (H) gellan gum, (I) starch, (J) shizophyllan, (K) pullulan, and (L) mannan.

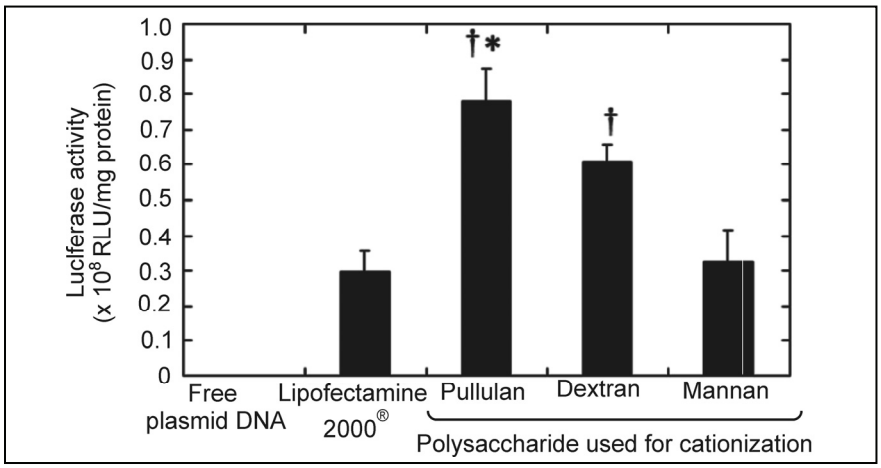


FIGURE 1.5 Effect of polysaccharide types on the expression level of luciferase plasmid DNA complex for MSC. *, p<0.05; versus the expression level of complexes prepared by other spermine-polysaccharides. †, p<0.05; versus the expression level of complexes prepared by Lipofectamine 2000® [65].

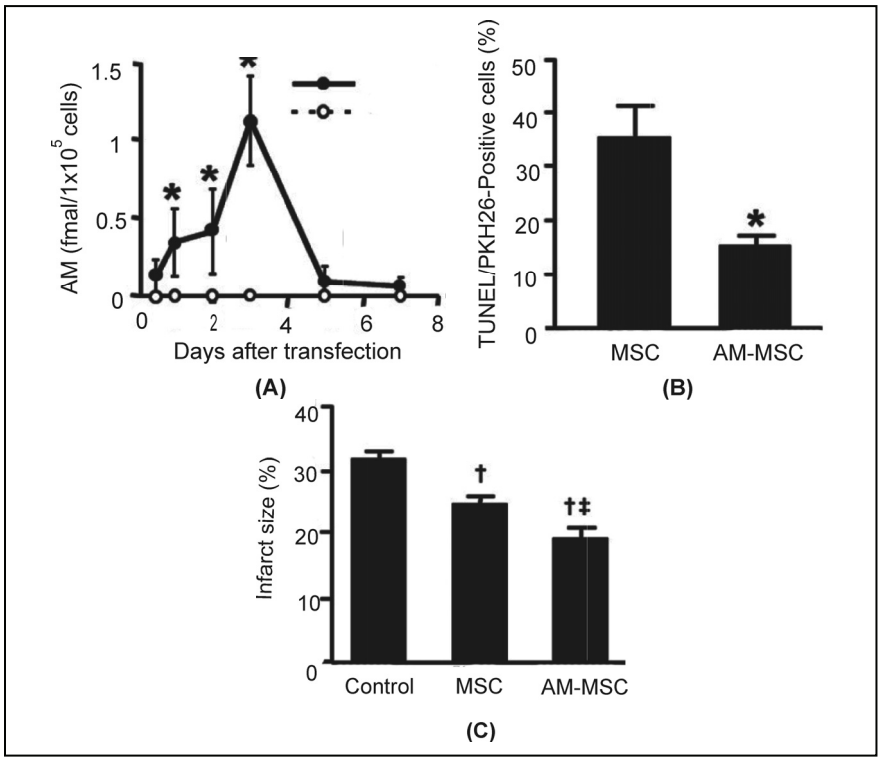
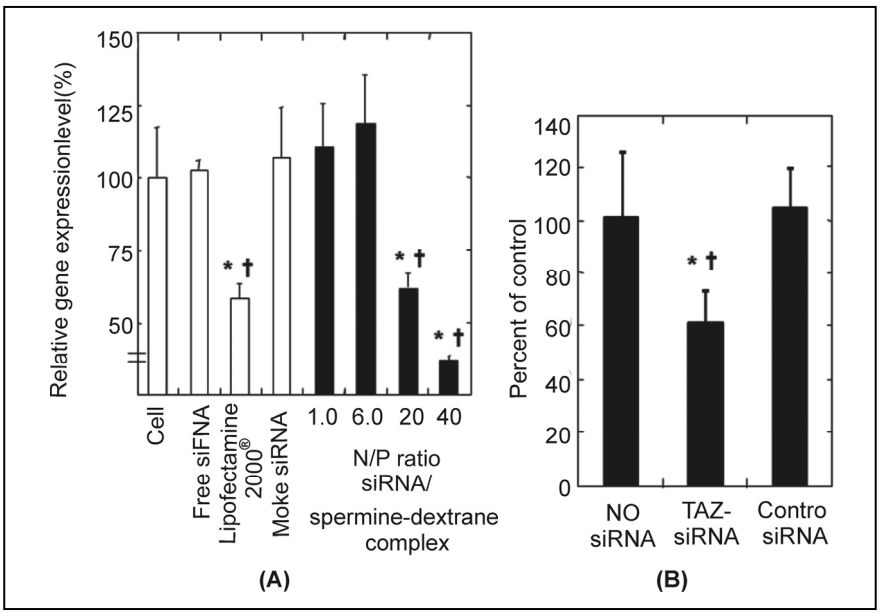


FIGURE 1.6 (A) Time course of AM secreted from MSC following the transfection by spermine-dextran-AM plasmid DNA complexes, AM-MSC. *, $p < 0.05$; versus the

level of original MSC at the corresponding time period. (B) *In vivo* anti-apoptotic effects of AM-MSC. Quantitative analysis of *in vivo* TUNEL assay for AM-MSC and MSC was performed. *, $p < 0.05$; versus the MSC. (C) Therapeutic effects of AM-MSC transplantation on the myocardial infarct size 4 weeks after coronary ligation. †, $p < 0.05$; versus the Control group. ‡, $p < 0.05$; versus the MSC group[66].

RNA interference (RNAi) has been recognized as a phenomenon that messenger RNA (mRNA) is sequence-specifically degraded to suppress the biological function of the corresponding protein[67]. Induction of this RNAi by a siRNA has been scientifically and therapeutically noted in cell biology. The siRNA-based mRNA-specific suppression will be able to artificially enhance or suppress the level of the subsequent gene expression, resulting in the biological manipulation of cell functions. We used spermine-dextran as a transfection carrier of siRNA for the gene suppression of MSC (Figure 1.7A)[68]. It has been reported that MSC are preferably differentiated into osteoblasts rather than adipocytes by a transcription coactivator containing PDZ-binding motif (TAZ) endogenously present[68]. Transfection of TAZ-siRNA complex with spermine-dextran (Figure 1.7B) enabled MSC to promote their differentiation into adipocytes (Figure 1.7C). This is a promising and new technology to control the differentiation direction of cells, which is different from the conventional methodology where the culture medium is modified.



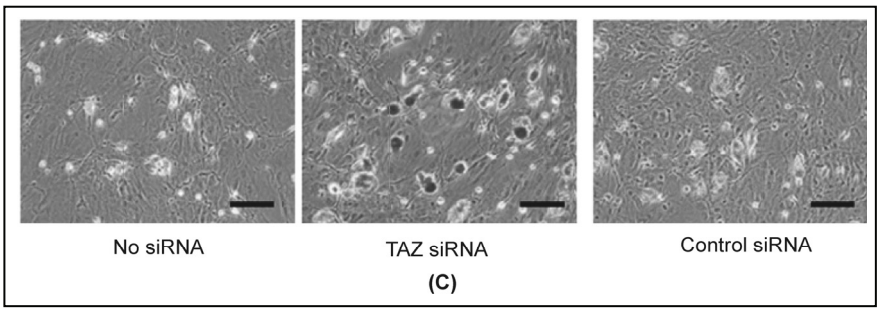


FIGURE 1.7 (A) Suppression effect of spermine-dextran-siRNA complexes on relative gene expression of MSC. As controls, free siRNA, siRNA for non-sense GFP, and Lipofecamine 2000-siRNA complex were used. Percent expressed for MSC without siRNA transfection (Cell) is 100%. *, $p < 0.05$; versus the expression percentage of MSC without siRNA transfection. †, $p < 0.05$; versus the expression percentage of MSC transfected with free siRNA. N/P ratio defines as the molar ratio of nitrogen molecule of spermine-dextran to the phosphorous ones of siRNA. (B) Level change in the TAZ mRNA expression of MSC transfected with spermine-dextran-siRNA complexes. Level of TAZ mRNA expression was evaluated 2 days after siRNA transfection. Amount of siRNA used for transfection is 10 pmole. Percent changed for MSC without siRNA transfection is 100%. *, $p < 0.05$; versus the change percentage of MSC without siRNA transfection. †, $p < 0.05$; versus the change percentage of MSC transfected with the complex of control-siRNA. (C) Phase-contrast microscopic pictures of MSC stained by oil red O. Cells were incubated for 12 days after siRNA transfection. The dose of siRNA used was 20 nM. Bar = 100 μm[68].

When cells are manipulated and used for transplantation therapy, in addition to the enhancement of transfection and expression of nucleic acid, it is undoubtedly important to consider the physiological and functional conditions of cells transfected from the viewpoint of the practical usage. In the conventional procedure of nucleic acid transfection, normally the non-viral carrier is complexed with a plasmid DNA, and then added to the culture medium of cells for transfection. In this case, although the presence of serum is essential to maintain the culture conditions of cells biologically good, the transfection culture is generally being carried out without the serum. This is because the plasmid DNA-carrier complex often interacts with serum components. This interaction often reduces the extent of complex internalized into cells, leading to the suppressed expression level of nucleic acid. Taken together, we cannot always say that the culture condition for the conventional transfection is good in terms of cells viability. Basically, there are two approaches to improve the culture conditions of nucleic acid transfection. One is the technical modification to perform the transfection of nucleic acid even in the presence of serum. The other is to improve the methodology of cell culture which enables cells to

physiologically proliferate under good conditions in the transfection culture with nucleic acid. Since a three-dimensional culture substrate has a large surface area available for cell attachment and the subsequent proliferation compared with a two-dimensional tissue culture plate, cells can be generally proliferated in the three-dimensional substrate at higher rates and for longer time periods than those in the two-dimensional one. Moreover, combination with a perfusion culture method can supply nutrients and oxygen to the cells proliferated in the three-dimensional (3D) substrate efficiently compared with a static culture method, while harmful metabolic products and wastes generated from cells can be excluded rapidly. It has been previously demonstrated that the proliferation of MSC was greatly influenced by their culture method and significantly enhanced by the perfusion culture method compared with the static method. Therefore, it is highly expected that combination of cationized polysaccharide-based transfection with the culture method enhances the expression level of nucleic acid to a great extent compared with the conventional method of nucleic acid transfection.

A new non-viral method of transfection was designed to enhance the expression level of nucleic acid for rat MSC. The spermine-pullulan was complexed with a plasmid DNA of luciferase and coated on the surface of culture substrate together with Pronectin® of artificial cell adhesion protein (Figure 1.8A)[70]. MSC were cultured and transfected on the complex-coated substrate (reverse transfection), and the level and duration of gene expression were compared with those of MSC transfected by culturing in the medium containing the plasmid DNA-spermine-pullulan complex (conventional method). The gene expression was enhanced and prolonged by the reverse transfection method to a significantly great extent compared with that of the conventional method (Figure 1.8B). The reverse method permitted the transfection culture of MSC in the presence of serum, in marked contrast to the conventional method, which gave cells a good culture condition, resulting in lower cytotoxicity (Figure 1.8C).

The reverse transfection can be carried out for 3D culture substrate from the viewpoint of transfection procedure. Non-woven fabic of polyethylene terephtalate (PET) coated with the complex and Pronectin® while the cell culture was performed by bioreactor systems, such as an agitated and stirring culture methods. The level and duration of gene expression for MSC were significantly enhanced by the two bioreactor methods compared with that of the static method (Figure 1.8D). It is possible that the medium circulation improves the culture conditions of cells in terms of oxygen and nutrition supply and wastes excretion,

resulting in an enhanced nucleic acid expression. These results strongly demonstrated that comparable to the research and development of non-viral carriers themselves, it is important to improve the technology and methodology of cell culture which give cells good conditions to maintain their vital and biological functions as well as efficiently enhance nucleic acid transfection[71].

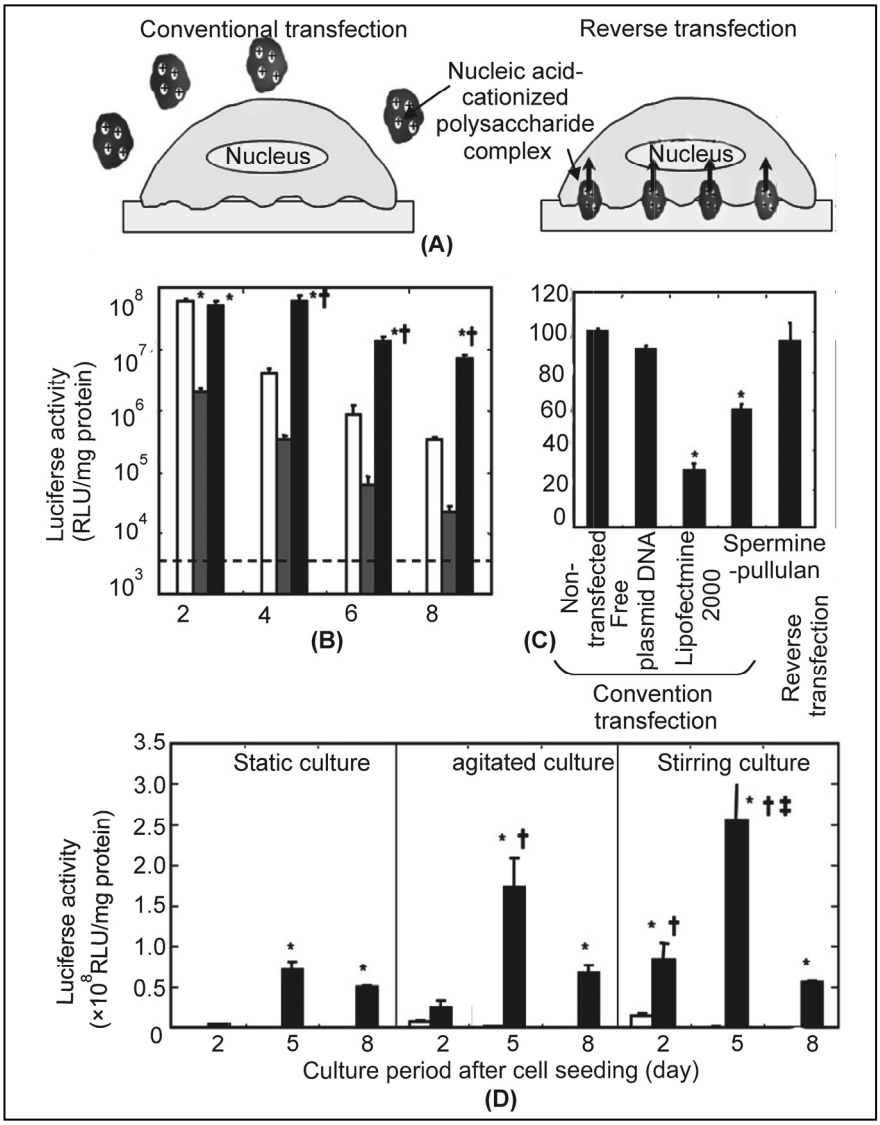


FIGURE 1.8 (A) Illustration of conventional or reverse gene transfection. (B) Time course of luciferase expression level of MSC transfected by the

conventional (open and light gray columns) and reverse methods (solid columns) in the static cuture: (open columns) the plasmid DNA-spermine-pullulan complex in the absence of fetal calf serum (FCS), (light gray columns) the plasmid DNA-spermine-pullulan complex in the presence of FCS, and (solid columns) the plasmid DNA-spermine-pullulan complex in the presence of FCS. The dotted line indicates the level of non-transfected, original MSC. *****, $p < 0.05$; versus the level in the presence of FCS by the conventional method at the corresponding time. †, $p < 0.05$; versus the level in the absence of FCS by the conventional method at the corresponding time. (C) Cell viability of MSC 2 days after conventional and reverse transfection cultures. The cells were transfected by the conventional method with free plasmid DNA or that complexed with Lipofectamine 2000 and spermine-pullulan in the absence of serum. The cells were transfected by the reverse method with plasmid DNA-spermine-pullulan complex in the presence of serum. *, $p < 0.05$; versus the cell viability of non-transfected, original MSC. (D) Time course of luciferase expression level of MSC transfected by the conventional (open columns) and reverse methods (solid columns) in the static, agitation, and stirring cultures in the PET non-woven fabric: (open columns) the plasmid DNA-spermine-pullulan complex in the absence of FCS and (solid columns) the complex in the presence of FCS. *, $p < 0.05$; versus the level in the presence of FCS by the conventional method at the corresponding time. **†**, $p < 0.05$; versus the level in the absence of FCS by the reverse method in the static culture at the corresponding time. **‡**, $p < 0.05$; versus the level in the absence of FCS by the reverse method in the agitation culture at the corresponding time[70].

1.3 Role of Biomaterials in Evaluation of Tissue Regeneration

It is indispensable to develop methodologies and technologies which enable to accurately and non-invasively evaluate the phenomenon and process of tissue regeneration. The distribution and biological function of cells and the ECM in the site to be regenerated should be revealed during the process of tissue regeneration process. There is no doubt that methodologies and technologies of molecular imaging play an important role in the evaluation of tissue regeneration. Molecular imaging is defined as the *in vivo* visualization of spatial and temporal distribution of molecular biological processes in the cell and tissue of interest[72]. The non-invasiveness of molecular imaging enables to repeatedly investigate the status of tissue regeneration induction in a patient, which gives more effective and active strategy individually from results obtained. Molecular imaging includes the imaging probes and the corresponding imaging modality. So far, various types of imaging probe have been reported, such as Gd^{3+}, Mn^{2+}, ^{19}F, and iron oxide nanoparticles for magnetic resonance imaging (MRI), radioisotopes (^{99m}Tc, ^{111}In, ^{123}I, ^{18}F, ^{64}Cu, and ^{124}I) or their derivatives for positron emission tomography (PET) and single photon emission computed tomography (SPECT),

luciferase and β-galactosidase for bio-luminescence imaging, and quantum dots and near-infrared fluorescent (NIRF) dyes for fluorescence imaging. There are two technologies required for the evaluation of tissue regeneration (Figure 1.9);

1. To sequentially trace the *in vivo* fate and biological function of cells transplanted and
2. To accurately visualize the phenomenon and process of tissue regeneration by using a specific molecule expressed or secreted during the regeneration process.

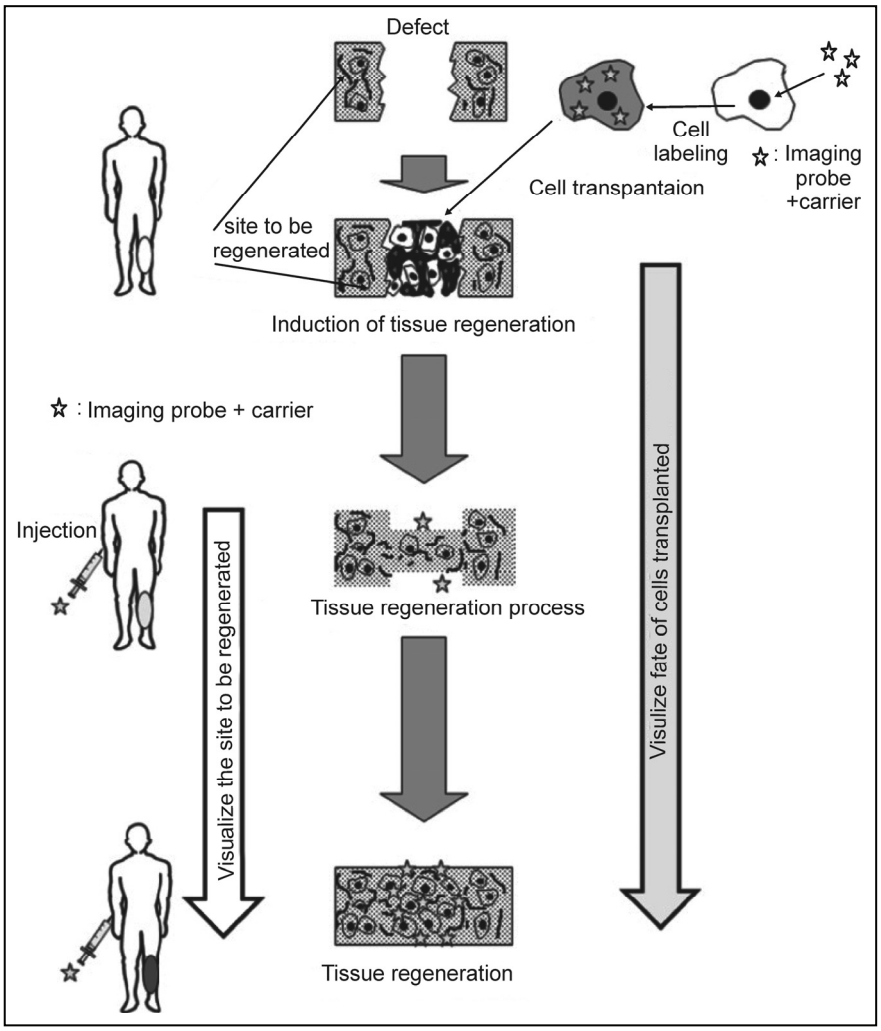


FIGURE 1.9 Technologies required for evaluation of tissue regeneration.

These can be achieved by the internalization of imaging probes into the cells (cell labeling) or their delivery to the site regenerated. Therefore, the DDS technologies of accelerated permeation and absorption or targeting of drug can be utilized to maximize the efficacy of molecular imaging by increasing in the ratio of signal to noise (S/N). It has been demonstrated that combination with nano-sized carriers, such as water-soluble polymers, polymer micelles, emulsions, and liposomes, allows the imaging probes to simultaneously condense and to stably deliver in the blood circulation, resulting in an enhanced imaging efficacy[73,74]. Furthermore, modification with targeting moieties such as antibody or targeting peptide enables nano-sized carriers to be actively delivered to the tissue of interest. On the basis of these findings, the nano-sized carriers incorporating imaging probes are highly expected to play an important role in the evaluation of tissue regeneration.

1.3.1 Polysaccharide as a Nano-sized Carrier for Cell Labeling

There have been many research trials on cell labeling technologies with imaging probe. The imaging probes used for cell labeling include iron oxide nanoparticles, several radioisotope derivatives, luciferase and β-galactosidase, quantum dots, and fluorescent dye (green fluorescence protein (GFP) etc.). In the case of the iron oxide nanoparticles or quantum dots, cell labeling is achieved by making use of transfection reagent, external stimulation or modifying the surface of particles suitable for cellular internalization. In the case of luciferase, β-galactosidase, and GFP, tranfection with the expressing plasmid DNA enable cells to visualize by bio-luminescence or fluorescence imaging methods. In the case of radioisotope derivatives, cell labeling is achieved by in advance transfection for cells with the plasmid DNA coding a protein which specifically interacts with a derivative and the subsequent incubation of the cells with the derivative.

Among the imaging probes for cell labeling, the iron oxide nanoparticles have been the most frequently used[75-77]. The iron oxide nanoparticles have been paid great attention as a material for biomedical

applications[78], such as a carrier for magnet responsive drug delivery system, a heating substance for hyperthermia, an adsorbent for magnetic separation and selection columns, and a negative contrast agent for MRI. MRI has been used for clinical diagnosis, while it is expected to be a molecular imaging modality with advantages of high spatial resolution and penetration depth. It is practically necessary for efficient cell labeling to accurately control the size and surface state of iron oxide nanoparticles. It has been well recognized that the iron oxide nanoparticles without any surface coatings often suffer from their aggregation in water or tissue fluid[79], resulting in the low labeling efficiency. Polymer coating enables iron oxide nanoparticles to make stable dispersion, and the iron oxide nanoparticles coated have been used in clinic. Since the iron oxide nanoparticles cannot be internalized into cells because of their surface nature of neutral or negative charge. Under these conditions, the cell labeling is performed by the combination of gene transfection agents or physical stimuli described above. However, such a labeling procedure is problematic in terms of its complexity and low efficiency. Therefore, a novel labeling procedure with simple and short-term incubation is highly expected. On the other hand, there are three important points for *in vivo* tracing of cell labeled; low cytotoxicity, maintenance of cells function, and long retention in the cells.

We designed iron oxide nanoparticles with different sizes and surface potentials by the conventional co-precipitation of ferric and ferrous ions in the presence of pullulan derivatives[80]. The size and surface potential of iron oxide-pullulan nanoparticles were changed by altering the mixing molar ratio of pullulan OH groups to ferric ions and the mixing percentage of pullulan derivatives, respectively. When MSC were labeled with the iron oxide-pullulan nanoparticles by the co-culture for 1 hr, the labeling efficiency and ^{1}H-MRI relaxivity were greatly influenced by the particle size and surface potential (Figures 1.10A and B), while the labeling procedure did not affect the viability (Figure 1.10C) and differentiation (Figure 1.10D) ability of MSC. These findings indicate that iron oxide-pullulan nanoparticle is a promising tool for the MRI labeling of MSC.

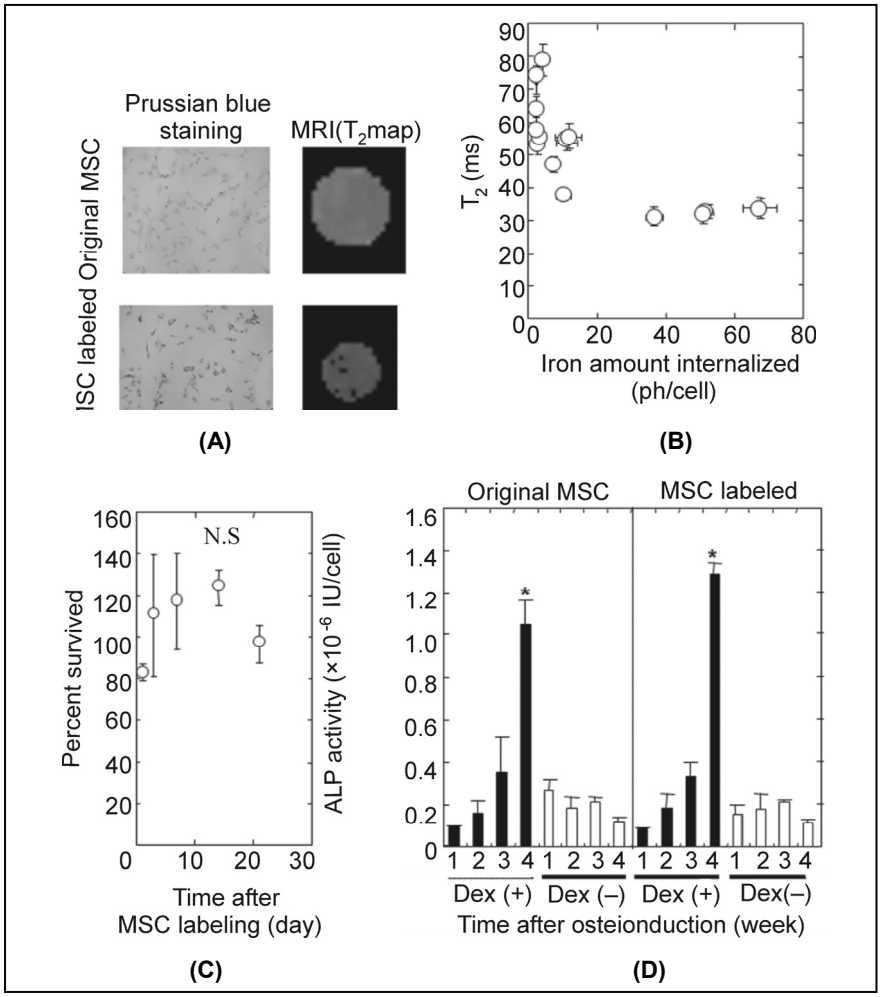


FIGURE 1.10 (A) Representative photographs of Prussian blue staining or quantitative T_2-map (MRI) for MSC labeled with or without iron oxide-pullulan nanoparticles. MSC are embedded in 10 wt% gelatin (200 μl) at a density of 1×10^6 cells/ml for MRI acquisition. (B) Relationship between the iron amount internalized and the T_2 relaxation time. (C) Time profile of viability of MSC labeled with iron oxide-pullulan nanoparticles. N.S.; not significant among times after MSC labeling. (D) Time profile of alkaline phosphatase (ALP) activity of original MSC or MSC labeled with iron oxide-pullulan nanoparticle in the medium containing 28 mM L-ascorbic acid, 10 mM β-glycerophosphate with (Dex (+)) or without 10 nM dexamethasone (Dex (-)). *, $p < 0.05$; versus the original MSC or MSC labeled with iron oxide-pullulan nanoparticles without induction of osteogenic differentiation at the corresponding time[80].

1.3.2 Polysaccharide as a Nano-sized Carrier for Targeting to the Regeneration Site

As described above, it has been demonstrated that nano-sized carriers are a powerful tool to effectively deliver imaging probes to the tissue of interest. There are a lot of research results about the visualization of tumor sites[81,82] or arteriosclerosis[83,84] by the injection of nano-sized carriers incorporating the related imaging probe. However, there are only a few researches on the visualization of regeneration site by making use of imaging probes combined with nano-sized carriers[85-88].

Polysaccharides and their derivatives have been paid great attention for drug delivery approaches because of their availability to improve the pharmacokinetical and pharmacodynamical characteristics of therapeutic agents, such as low molecular weight drugs, bioactive peptides or proteins, enzymes, and plasmid DNA. The chemical or physical conjugation of therapeutic agents with the polysaccharide derivatives increases their biological stability in the blood circulation, and consequently prolong the time period of activity. On the other hand, polysaccharides have been used to target the agents to the tumor (passive targeting) or the liver (active targeting). In addition, paramagnetic substance-loaded or radiolabeled polysacchrides have been applied for diagnostic imaging. Therefore, it is highly expected that imaging probes conjugated with the polysaccharide derivatives enhance the visualization efficacy in the site to be regenerated.

Angiogenesis is defined as the generation of capillaries from natural vessels and is a fundamental process involved in various phenomena including development, wound healing, tissue regeneration (physiological angiogenesis), and the progression of chronic inflammation and tumor (pathological angiogenesis)[89]. Angiogenesis occurs via several processes:

(i) the degradation of extracellular matrix surrounding the existing vasculature;
(ii) the proliferation and migration of endothelial cells thereat as well as the attraction of blood-derived macrophages and circulating stem cells; and
(iii) the integration of endothelial cells, followed by tube formation[90].

The hypoxia-inducible factor (HIF)-1α, vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP), and $\alpha_v\beta_3$ integrin have been used as a molecule of interest. Currently, the clinical trial on the induction therapy of angiogenesis is being performed for patients with ischemic diseases by cell transplantation or administration of bioactive

substances based on the controlled release system of gelatin hydrogels[91]. However, often the angiogenesis is not detected angiographically although the disease appearance of patients got better. This is mainly because there are no diagnostic systems to clinically detect blood vessels with a small diameter regenerated.

A new polysaccharide-based imaging probe have been designed and prepared for the MRI evaluation of therapeutic angiogenesis[92]. The probe consists of dextran, diethylenetriaminepentaacetic acid (DTPA) residue of a chelator, Gd^{3+}, and cyclic peptide containing an arginine-glycine-aspartic acid sequence (cRGD) with an inherent affinity for the $\alpha_v\beta_3$ integrin (cRGD-dextran-DTPA-Gd, Figure 1.11A). The cRGD-dextran-DTPA-Gd had an affinity for cells expressing the $\alpha_v\beta_3$ integrin and showed a higher longitudinal relaxivity compared with the DTPA-Gd of MRI contrast agent clinically used. Right femoral, external iliac, and deep femoral and circumflex arteries and veins were surgically ligated to prepare a mouse model of hindlimb ischemia. A laser Doppler analysis and histological evaluation experimentally confirmed that the hindlimb ischemia was naturally healed accompanying angiogenesis, while the $\alpha_v\beta_3$ integrin was expressed in the ischemic-angiogenic region without any treatment. When intravenously injected into mice with hindlimb ischemia, the cRGD-dextran-DTPA-Gd were significantly accumulated in the ischemic-angiogenic region and showed an MR ability to detect the ischemic-angiogenic region (Figures 1.11B-D).

Non-invasive imaging technologies have been widely used in clinical diagnosis. However, each imaging modality is based on quite different principles, and has the advantages and disadvantages. Generally, a single technique does not always correspond to all the requirements for diagnosis imaging[93]. As one trial to tackle the issue, it is practically possible to design a multimodal imaging system. A combinational imaging system composed of different imaging modalities may compensate the deficiencies of single imaging modality, while it gives useful and new tools to biomedical researches and clinical diagnosis. Currently, some prototypes of multimodal imaging system including MRI–optical, NIRF–SPECT, PET-CT, and SPECT-MRI, have been introduced. However, the research and development of multimodal imaging probes are still in an early stage although they are highly required to realize the idea of multimodal imaging. To design and prepare such a multimodal imaging system, it is also of prime necessity to make the best of DDS technology to enhance the accumulation of imaging agents in the target tissue, resulting in the increased S/N ratio.

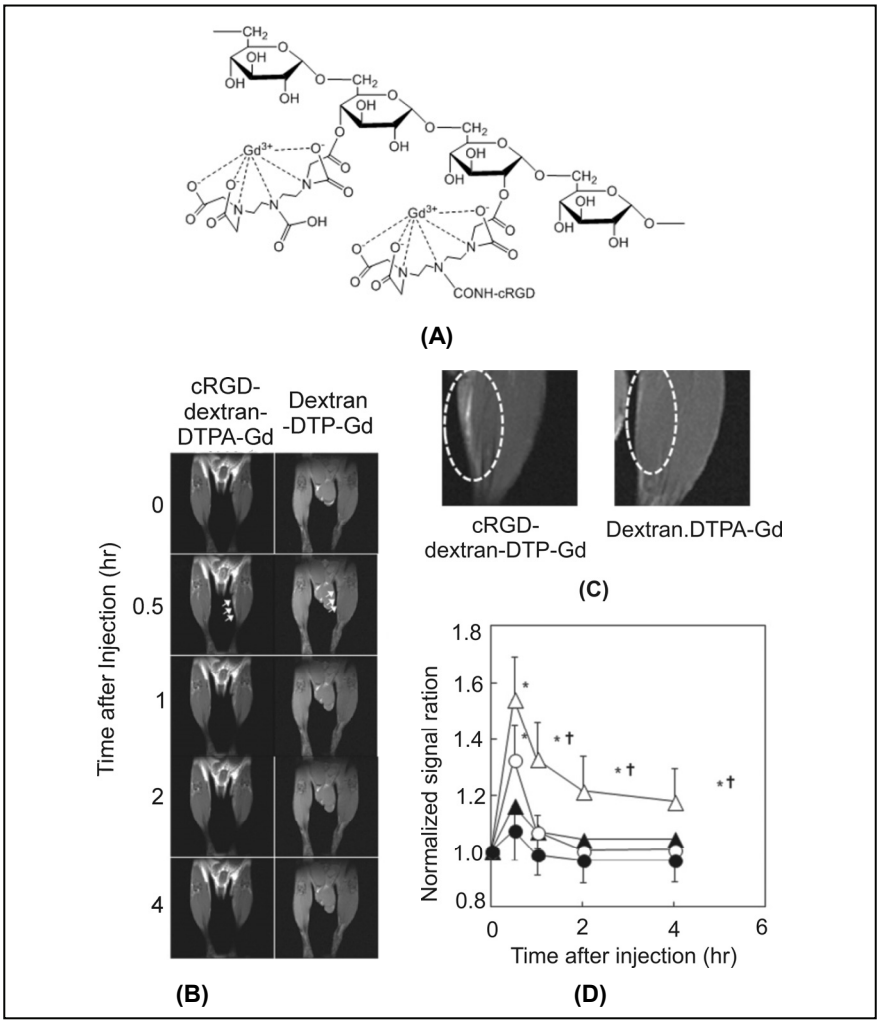


FIGURE 1.11 (A) Chemical structure of cRGD-dextran-DTPA-Gd. (B) Representative MRI pictures of mice hindlimb region before and after injection of dextran-DTPA-Gd or cRGD-dextran-DTPA-Gd. The right side shows the ischemic hindlimb. Arrows indicate the ischemic-angiogenic region. (C) Representative enlarged image of ischemic-angiogenic region (dashed ellipses). (D) Time profiles of T_1 signal intensity ratio in the mice hindlimb after injection of dextran-DTPA-Gd (○, ●) or cRGD-dextran-DTPA-Gd (△, ▲). Signals (5 points each) were acquired in normal (solid) and ischemic-angiogenic region (open). *, $p < 0.05$; versus the normalized signal ratio in the normal region after the injection of corresponding agent at the corresponding time. †, $p < 0.05$; versus the normalized signal ratio in the ischemic-angiogenic region after injection of dextran-DTPA-Gd at the corresponding time[92].

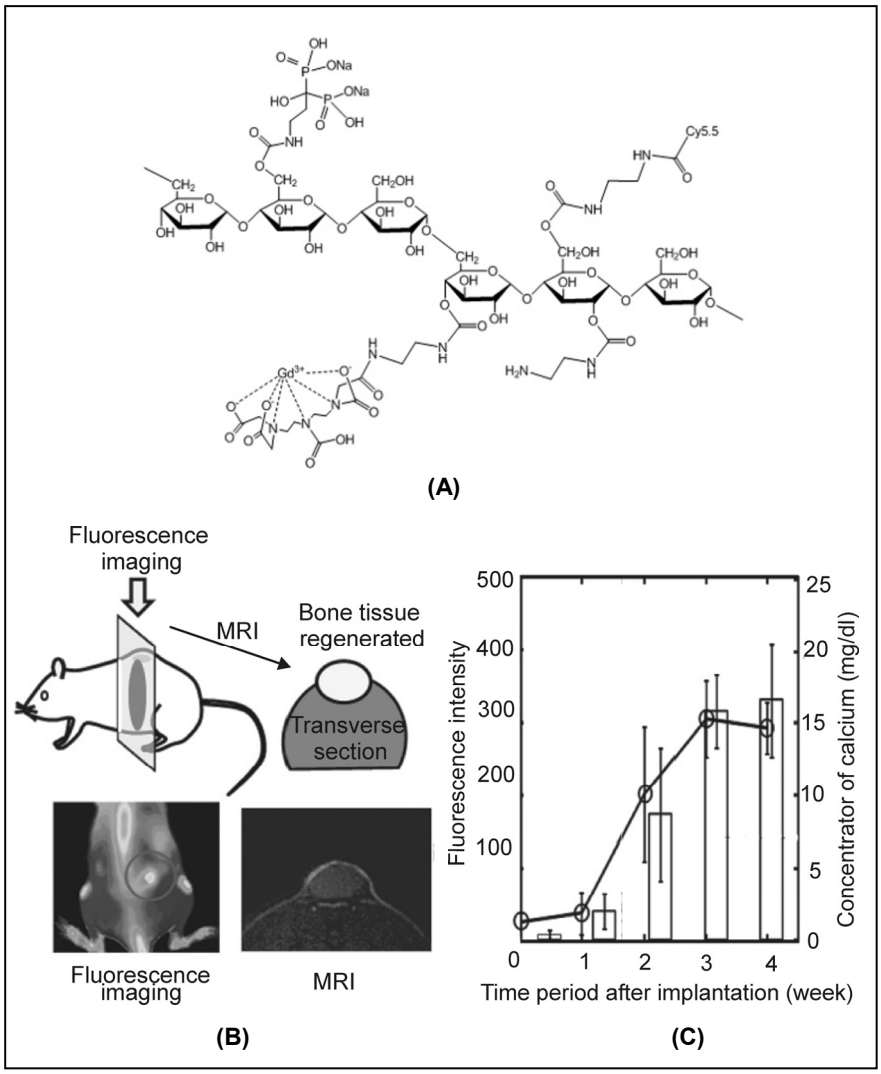


FIGURE 1.12 (A) Chemical structure of PA-pullulan-F/M. (B) Representative pictures of mice injected with PA-pullulan-F/M acquired by fluorescence imaging or MRI. Injection was performed for mice 3 weeks after the hydrogel implantation. Site implanted with hydrogel incorporating BMP-2 is indicated by a red circle for the fluorescence image. Trans-axial multi-slice T_1-weighted MR images were repeatedly acquired with a spin-echo sequence. (C) Time profiles of calcium concentration of subcutaneous tissues around gelatin hydrogels incorporating BMP-2 implanted (open bars) and fluorescent intensity of the site around gelatin hydrogels incorporating BMP-2 implanted (open circles)[96].

Generally, bone healing and repairing have been diagnosed so far by CT, X-ray, and MRI, and their methodology has obtained the clinical reliability. However, the convention imaging methods cannot always give us detailed information about soft and bone tissues regenerated even in the early stage. Although several researches have been performed on bone-specific imaging probes[94,95], few multimodal imaging probes are developed. If a multimodal imaging system to visualize the regeneration process of bone tissues can be developed, the extent of bone regeneration and repairing will be more clearly diagnosed and consequently become a new therapeutic strategy with high reliability. In addition, the system can also give an evaluation method to observe whether or not the process of bone tissue regeneration takes place properly. For this purpose, the modality combination of MRI with a superior property of tomography as well as a high spatial resolution and optical imaging with a high sensitivity and specificity would be a promising choice.

A new polysaccharide-based imaging probe have been designed and prepared for multimodal imaging system for the evaluation of bone regeneration processes[96]. The polysaccharide-based imaging probe consists of pullulan, DTPA, Gd^{3+}, Cy5 of fluorescent dye, and pamidronate (PA) of bisphosphonates with a high affinity for hydroxyapatite (PA-pullulan-F/M, Figure 1.12A). The PA-pullulan-F/M had an affinity for hydroxyapatite and showed an MRI ability similar to Gd-DTPA clinically used. A gelatin hydrogel incorporating bone morphogentic protein (BMP)-2 was prepared and implanted subcutaneously into mice to obtain an animal model of bone regeneration[97]. When intravenously injected into mice with the bone tissue ectopically formed by the BMP-2-incorporated hydrogel to evaluate their body distribution by the fluorescence imaging and MRI, the PA-pullulan-F/M accumulated in the bone tissue regenerated (Figure 1.12B). It should be noted that the time profile of fluorescent intensity well corresponded with that of calcium amount in the bone tissue newly formed (Figure 1.12C). These findings clearly indicated that the PA-pullulan-F/M is a useful multimodal imaging probe which enables to evaluate not only the phenomenon, but also the process of bone regeneration.

Conclusion

This chapter described the role of polysaccharides in the induction and evaluation of tissue regeneration and summarized the present status of

related researches. The advantages of polysaccharide as a material include the ease of acquisition, chemical modification, and construct formation. With the development of "Glycoscience", the novel biological functions of polysaccharides are being revealed one after another. It is no doubt that innovative polysaccharide constructs for biomedical applications containing tissue regeneration technologies will be created by actively making use of these functions. The tissue regeneration is interdisciplinary research field. To achieve effective induction and evaluation using polysaccharide-based technologies, substantial collaborative researches between material, pharmaceutical, image technological, biological, and clinical scientists are highly required. Such researchers must have knowledge in medicine, biology, pharmacology, image technology in addition to material sciences. We will be happy if this chapter stimulates readers' interest in the research field of tissue regeneration and assists their understanding of importance in induction and evaluation of tissue regeneration with polysaccharide-based cell scaffold and DDS.

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