Advanced Drug Delivery Reviews 160 (2020) 1–18

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Repairing the heart: State-of the art delivery strategies for biological therapeutics

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ARTICLE INFO

Article history:
Received 5 June 2020
Received in revised form 1 October 2020
Accepted 3 October 2020
Available online 8 October 2020

Keywords:
Cardiac repair
Synthetic delivery approach
Protein delivery
Nucleic acid delivery
Modified mRNAs

ABSTRACT

Myocardial infarction (MI) is one of the leading causes of mortality worldwide. It is caused by an acute imbalance between oxygen supply and demand in the myocardium, usually caused by an obstruction in the coronary arteries. The conventional therapy is based on the application of (a combination of) anti-thrombotics, reperfusion strategies to open the occluded artery, stents and bypass surgery. However, numerous patients cannot fully recover after these interventions. In this context, new therapeutic methods are explored.

Three decades ago, the first biologicals were tested to improve cardiac regeneration. Angiogenic proteins gained popularity as potential therapeutics. This is not straightforward as proteins are delicate molecules that in order to have a reasonably long time of activity need to be stabilized and released in a controlled fashion requiring advanced delivery systems. To ensure long-term expression, DNA vectors-encoding for therapeutic proteins have been developed. Here, the nuclear membrane proved to be a formidable barrier for efficient expression. Moreover, the development of delivery systems that can ensure entry in the target cell, and also correct intracellular trafficking towards the nucleus are essential. The recent introduction of mRNA as a therapeutic entity has provided an attractive intermediate: prolonged but transient expression from a cytoplasmic site of action. However, protection of the sensitive mRNA and correct delivery within the cell remains a challenge. This review focuses on the application of synthetic delivery systems that target the myocardium to stimulate cardiac repair using proteins, DNA or RNA.

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https://doi.org/10.1016/j.addr.2020.10.003
0169-409X/

1. Introduction

1.1. Cardiac repair

Ischemic cardiovascular diseases are still the leading cause of mortality in the world. Myocardial infarction (MI) accounts for almost half of all deaths due to cardiovascular diseases [1]. MI refers to the process of cell apoptosis and necrosis that occurs after the acute or chronic blockage of a coronary artery supplying blood to a particular region of the myocardium. After MI, the injured cardiac cells will be replaced by scar tissue to maintain organ integrity and prevent ruptures since the cardiomyocytes in the adult human heart have a very limited regenerative capacity. Consequently, the damaged heart develops further fibrosis and cardiac dilatation, eventually resulting in fatal heart failure in ~30% of cases. Nowadays, clinical therapies for MI mainly concentrate on pharmacological treatment (such as thrombolytics, aspirin, angiotensin-converting enzyme inhibitors, β-blockers) and/or surgical interventions (percutaneous coronary intervention and coronary artery bypass surgery), which has improved survival and quality of life for those suffering from MI. However, these fail to retain cardiac function in the long term, and a significant number of patients will develop a chronic state of heart failure, eventually [2–7].

Therefore, stimulation of cardiac repair and ultimately regeneration to rescue the ischemic myocardium and prevention of disease progression is a significant challenge. It is believed that terminally differentiated cardiomyocytes in adult mammalian hearts have exited the cell cycle and have minimal ability to proliferate after MI [8]. However, a recent study revealed that progressive systemic hypoxia can trigger the intrinsic regenerative ability in the adult mouse heart, which strongly challenges the common conception that existed for many years [9]. Also, new cardiomyocytes can be detected in the human adult heart which also points to a certain degree of regenerative potential [10]. Quantitative studies indicate that cardiomyocytes do renew themselves, but their regeneration potential decreases over time. It reduces from 1% replacement rate each year at the age of 25 to 0.45% at the age of 75 [10]. This means that no more than half of the cardiomyocytes can be replenished during life. On the one hand, these studies show that it is possible for the heart to regenerate, but on the other hand, however, it is clear that this endogenous level of renewal is insufficient to repair the myocardial injury after MI. This emphasizes the need for research into the stimulation of the reparative and ultimately regenerative process. Efficient delivery systems that can deliver DNA, RNA or proteins with specific functions are lacking but fulfill a crucial role to stimulate the repair of the injured heart. Although different appropriate biological signals have been identified, with specific needs, pros and cons, they all need advanced systems to deliver the therapeutics in a precise and consistent manner for the regenerative process to occur correctly (Fig. 1).

1.2. Cardiac delivery

Another challenge in successful cardiac repair is effective cardiac delivery of these biological macromolecules such as proteins, DNA and mRNAs into the diseased domain (Fig. 2).

Depending on the delivery route, the number of barriers they have to pass are different. For systemic delivery, the therapeutic has to survive in the circulation and reach the diseased tissue, in this case the infarcted area of the heart. This involves local extravasation from the circulation into the myocardium. For certain proteins, e.g. growth factors, which function by receptor-binding on the cell surface this arrival in the tissue may already be enough. For DNA, mRNA and some proteins tissue localization alone is not sufficient as these therapeutics need to arrive intracellularly to be functional. As spontaneous membrane translocation is virtually impossible for these macromolecules they need to enter the cell through endocytosis and successfully escape before *endo*-lysosomal degradation.

For local delivery, either by intramyocardial injection or application on the surface of the myocardium, therapeutics have immediate access to the diseased tissue but also then, intracellular activity can be a major hurdle. Besides these general challenges for protein, DNA and mRNA therapeutics for cardiac repair, each modality has specific advantages and disadvantages of their own (Table 1).

In most of the biological processes, proteins are the ultimate workhorse. Thus, direct protein delivery is a very attractive approach. However, they do have relatively short half-lives, need repeated administration and every protein requires tailored strategies. Gene therapy, on the other hand, is a promising technology to ensure long-term expression, but appropriate vehicles are required for effective myocardial therapy. Currently, there are two main types of gene delivery carriers: viral and non-viral gene vectors. Non-viral gene delivery systems have attractive properties over viral vectors. They always contain drugs have similar properties, have low immunogenic properties, and unlimited DNA size for loading, a relatively low cost of production and low toxicity [11]. The development of non-viral systems are rapidly progressing and some non-viral synthetic carriers, such as cationic polymers and ionizable lipid nanoparticles, have shown early successes in gene delivery from cell membrane to the nucleus [12]. One of the challenges to using plasmid DNA for gene therapy is its need to enter into the nucleus to be expressed, which is very difficult for terminal differentiated non-proliferating organs such as the heart. Recently developed modified mRNA provides durable transient expression but does not require to go to the nucleus to be translated.

This review provides a perspective on therapeutic approaches using genes, mRNA and proteins for cardiac repair using synthetic delivery systems. The different approaches are summarized in Table 2 and discussed in the respective sections.

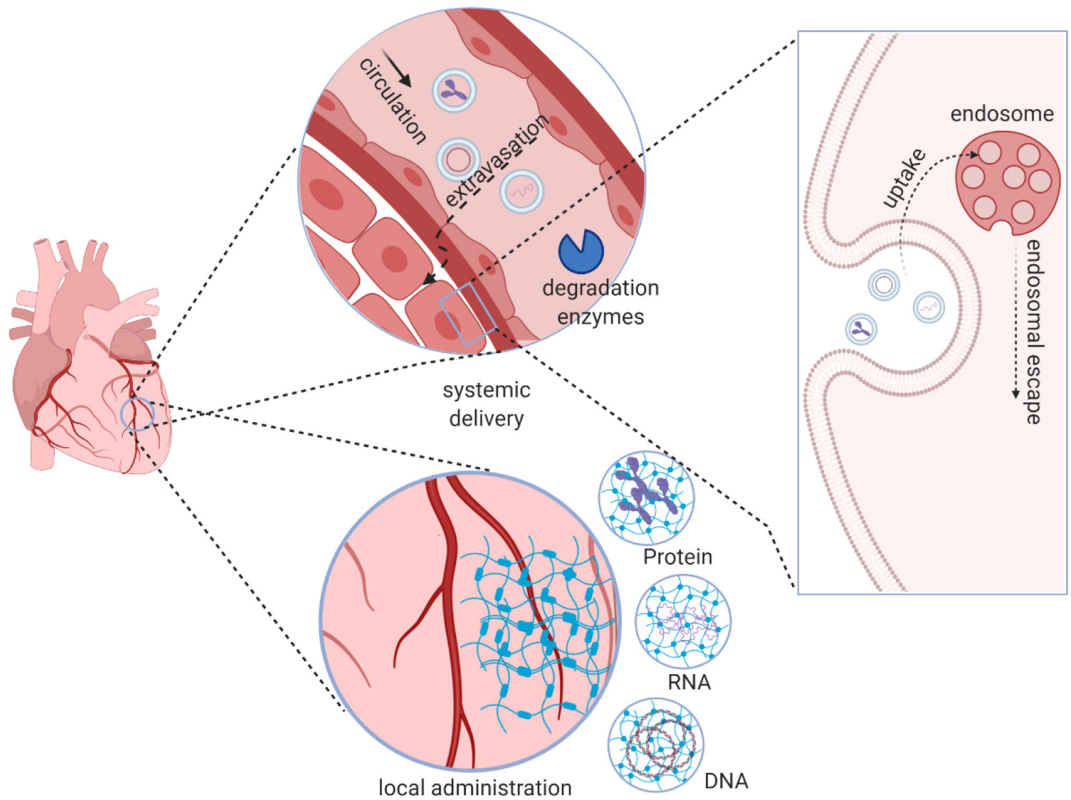

Fig. 1. Challenges in cardiac delivery of biological macromolecules. For system delivery, avoiding non-target tissue clearance and degradation while reaching the diseased tissue, through extravasation into the infarcted area of the heart are important obstacles. For local delivery, intramyocardial injection or application on the surface of the myocardium with a cardiac patch or gel offers immediate access. For DNA, mRNA and some proteins with an intracellular site of activity the most important barrier can be overcoming the cellular membranes to arrive in the cytoplasm. Uptake by endocytosis and successful escape from the *endo*-lysosome before degradation are key aspects.

2. Protein delivery systems

Proteins are the workhorses of the cell and, however, are not always the first choice as therapeutics. There are many potential challenges to use protein as therapeutics directly, which include the costly production of protein, difficulties to produce them with right tertiary structure and appropriate posttranslational modifications and their deliver to the heart. Localized and sustained angiogenic growth factor delivery into ischemic myocardium has improved angiogenesis, cardiomyocyte survival and proliferation, and an overall reduction in cell apoptosis and cardiac remodeling [55]. However, clinical translation of simple protein delivery failed, and therefore, high doses of these proteins may be needed, which could induce side effects, such as promoting tumor growth. This underlines the careful balance between on and off-target effects that need to be found for proteins, moreover, proteins are usually susceptible to proteolysis and are often rapidly cleared. To overcome these drawbacks, there has been considerable interest in establishing delivery platforms that are capable of protecting and controlling the release of therapeutic proteins for cardiac regeneration.

2.1. Hydrogels-based protein delivery systems

Hydrogels appear to be an ideal candidate for biomedical applications due to their biocompatible properties for intramyocardial injection. The aqueous environment inside the gel allows many proteins to have their preferred conformation.

A variety of studies investigated natural hydrogels. VEGF is one of the most popular proteins for cardiac repair, given its pivotal role in angiogenesis. Nevertheless, VEGF's extremely short half-life and potential side-effects make it very challenging for clinic translation [88,89]. It was shown that repeated infusions with VEGF or via controlled slow-release achieved a better response in patients compared to a single bolus [90]. Prolonged exposure produced favorable effects at low plasma levels, which lowers the risks related to VEGF overdosing. This provided a strong rationale for hydrogel-mediated extended VEGF delivery. As an example, Dwyer et al. fused VEGF with polyglutamic acid polypeptides, and encapsulated it into a hyaluronic acid hydrogel. This hydrogel is biocompatible and offered a prolonged release of VEGF for more than one month [91]. Two other studies also showed that VEGF-release from a biodegradable hydrogel could promote angiogenesis and cardiac function in a rat model of acute myocardial infarction [14,13].

Promoting the proliferation of endogenous cardiomyocytes in the border zone of the infarcted area can be an effective strategy for cardiac repair following MI. 6-Bromoindirubin-3-oxime (BIO) and Insulin growth factor-1 (IGF-1) are two potential factors that can stimulate cardiomyocyte survival and proliferation. Fang et al. performed a study in an MI rat model on the controlled delivery of BIO and IGF-1 in a combined gelatin-based hydrogel system to stimulate cardiac repair. The proliferation of cardiomyocytes and revascularization around the infarcted areas was seen, which eventually contributed to an improved cardiac function [30]. Similarly, injection of an alginate hydrogel, incorporating IGF-1 and HGF, has demonstrated to boost cardiomyocytes' survival, attenuate adverse cardiac remodeling and promote cardiac function in a rat model of acute MI [92]. Awada et al. encapsulated tissue inhibitor of metalloproteinases-3 (TIMP-3), fibroblast growth factor-2 (FGF-2) and stromal cell-derived factor-1α (SDF-1α) simultaneously, thereby improving cardiac contractility, reducing ventricular dilation, inflammation, fibrosis and extracellular matrix degradation in a rat model of myocardial infarction [93].

Hepatocyte growth factor (HGF) has anti-fibrotic, pro-angiogenic and cardioprotective effects. However, its clinical application is limited due to high instability and costs. Recently, HGF fragment (HGF-f), was

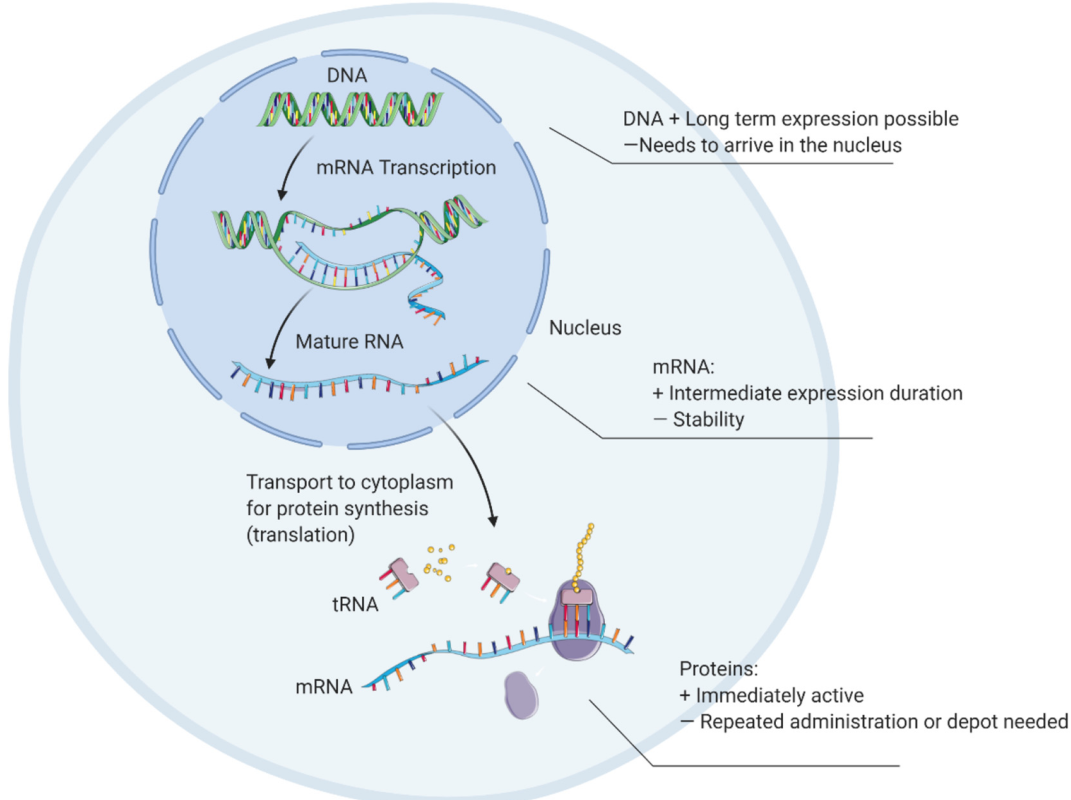

Fig. 2. The advantages and disadvantage of different therapeutics. Administering proteins to the damaged heart has the advantage that proteins are immediately active, but activity over a prolonged period of time requires controlled release from a depot or frequent administration. An alternative is the administration of the encoding DNA, which enables long-term expression. Here the major hurdle is the nuclear membrane that the DNA needs to cross in order to be active. An intermediate choice is using encoding mRNA. This enables durable expression that is still transient from a cytoplasmic site of action. A drawback is the intrinsic instability of mRNA.

designed to have augmented stability and activity. Two types of hydrogels were used, one based on extracellular matrix and one based on collagen, to promote the sustained release of HGF-f in a rat myocardial infarction model. The extracellular matrix-based hydrogel improved adverse left ventricular remodeling and increased arteriole density [32].

Although the use of extracellular matrix-based hydrogels can be used for different therapeutic molecules as well, including microRNAs and Extracellular vesicles [94], the applications of exogenous natural hydrogels in clinical studies remain, however, debated because of their immunogenicity and challenging quality control over physical properties and degradation rate.

In this regard, synthetic hydrogels may offer a better alternative. The drug release and degradation rate can be manipulated by regulating the density of crosslinking and swelling [95,96]. Hybrid systems of natural and synthetic systems include hyaluronate modified with hydroxyethyl methacrylate for cross-linking [97]. Stromal cell-derived factor-1α (SDF-1α) was mixed with a radical polymerization initiator, then injected intra-myocardially in a rat MI model, where gelation occurred. The result showed beneficial outcomes on ejection fraction, capillary density, and contractility in comparison with the controls group.

The delivery of synthetic hydrogels to the injury heart has shown to be effective in alleviating cardiac apoptosis, fibrosis and infarcted area, as well as improving angiogenesis and cardiac function using simple molecules like colchicine or complex structures like extracellular vesicles In different models of MI including rat and mouse [98–100]. Interestingly, the timing of administration can be an essential factor for success. An administration immediately after infarction was not beneficial, whereas improved remodeling and cardiac function can be observed when the hydrogel was injected one week after the establishment of rat MI model [101].

In addition to VEGF, basic fibroblast growth factor (bFGF) is another well-known angiogenic factor. Also this factor has therapeutic potential for MI, which is also limited by a relatively short half-life. bFGF was encapsulated into a dextran-grafted poly(*E*-caprolactone)-2-hydroxylethyl methacrylate/poly(N-isopropylacrylamide (Dex-PCL-HEMA/PNIPAAm) hydrogel. The effects on angiogenesis and cardiac function were assessed in a rat model of acute MI. Reduced infarct area and cardiac remodeling, enhanced angiogenesis and cardiac

Table 1
The advantages and disadvantages of different therapeutic modalities.

	Advantages	Disadvantages
Protein	Easy to produce Immediately effective Can function extracellularly	Costly Short half-life Repeated administration Possible immunogenicity
DNA	Easy to produce Can have long-lasting effect	Generally low expression Requires special delivery methods Needs to enter the nucleus
mRNA	Easy to produce Has intermediate-lasting effect No need to enter nucleus	Requires special delivery methods
Extracellular vesicles/EVs	Natural vector Low immunogenicity Can function either outside or inside the cell Has multimodal mechanism of action	Costly production Difficult quality control Challenge to load cargoes

Table 2
Synthetic approaches that have been used for cardiac repair.

Vector	DNA	RNA	Protein
Hydrogel			VEGF [13–16] VEGF/PDGF [17] VEGF/HGF [18] FGF-2 [19–23] bFGF [24–29] IGF-1 [30] IGF-1/HGF [31] HGF [32] HGFdf [33,34] Mydgf [35] MGF [36]
Nanoparticles/microparticles	HGF 44 [37] VEGF [38,39] HGF [37]	erythropoietin mRNA [40] GFP mRNA [41] AMO-1 [42] SHP-1 siRNA [43] IGF1 mRNA [44] nuclease mRNA [45] PTEN mRNA [46] VEGF siRNA [47] antagomiR-92a [48]	VEGF [39,49] VEGF/VEGF [50] IGF [51] VEGF [52] FGF-1 [53] VEGF [54,55] FGF-1 [56] FGF-2 [57] bFGF/IGF-1 [77]
Patch/Scaffolds			VEGF [58,59] MGF [60] IL-10 [61] IGF-1/SDF-1/VEGF [62]
Lipid based particles	VEGF [76]	VEGF-A mRNA [63] miRNA-181b [64]	VEGF [49,65,66] Erythropoietin [67]
Naked nucleic acid	VEGF [38,39,68–72]	VEGF [73]	
UTMD	VEGF [74–76] AKT [77] Ang-1 [78] GDF11 [79] SDF-1α [80] HGF 44 [81,82] TIMP3 [83] TB 4 [84]	ICAM-1 siRNA [85] single stranded oligonucleotides [86]	Cxcl12/Bmp2 [87]

Note: VEGF: Vascular endothelial growth factor; PDGF: Platelet-derived growth factor; HGF: Hepatocyte growth factor; FGF-2: Fibroblast growth factor-2; bFGF: Basic fibroblast growth factor-2; IGF-1: Insulin-like growth factor-1; HGFdf: Dimeric fragment of hepatocyte growth factor; Mydgf: Myeloid-derived growth factor; MGF: Mechano growth factor; FGF-1: Fibroblast growth factor-1; SHP-1: Src homology region 2 domain-containing tyrosine phosphatase-1; PTEN mRNA: Phosphatase and tensin homolog deleted on chromosome ten mRNA; IL-10: Interleukin 10; SDF-1: Stromal cell-derived factor-1; CXCL12: C-X-C Motif Chemokine Ligand 12; Bmp2: Bone morphogenetic protein 2; ICAM-1: Intercellular cell adhesion molecule-1; Ang-1: Angiopoietin-1; GDF11: Growth differentiation factor 11; TIMP3: TIMP Metallopeptidase Inhibitor 3; TB 4: Thymosin beta 4.

function were observed compared with the injection of either bFGF or hydrogel alone [25]. Similar results were observed by Garbern et al. [19] using the same hydrogel, yet a different therapeutic protein. In this case, high mobility group box 1 protein, a nuclear protein that serves as an architectural chromatin-binding factor, was used. HMGB1 encapsulated in a Dex-PCL-HEMA/PNIPAAm hydrogel, attenuated cardiac remodeling and improved cardiac function after rat MI [102].

Myeloid-derived growth factor (MYDGF), a newly verified paracrine-acting protein, has demonstrated a beneficial effect after MI [103]. This result has attracted great interest in exploring MYDGF as a therapeutic target for cardiac repair. Due to the low bioavailability of MYDGF, continuous infusion is needed to sustain the therapeutic protein levels. In order to maintain the local therapeutic level in the infarcted area during extended periods, an injectable, polyester hydrogel was developed from which the encapsulated MYDGF can be continuously released. Markedly decreased infarct size, improved wall thickness, neovascularization and heart function was noted in a rat MI model [35].

A challenging aspect of translating hydrogels into the clinic is their viscoelastic mechanical properties, which makes it very difficult to use catheter delivery for injection. Several strategies to circumvent this are in situ gelations [97] where the crosslinking occurs inside the myocardium or inclusion of poly(N-isopropylacrylamide) moieties that induce gelation at body temperature [25].

A novel method to obtain a catheter-deliverable hydrogel is to use shear-thinning material, i.e. a lipid-modified hyaluronate combined with poly-ethylene glycol-poly lactic acid microparticles. This material was well injectable. HGFdf and SDF-1α were encapsulated and the hydrogel was tested in a myocardial ischemia model in rats. Favorable biological and functional effects were seen, also when this therapy was tested in a large animal model, i.e. sheep myocardial ischemia [34]. Finally, a fast pH-switchable and self-healing supramolecular hydrogel carrier were developed to allow local catheter injection into the myocardium of a chronic pig myocardial infarction model. Sustained delivery of IGF/HGF in this UPy hydrogel resulted in the formation of new, small cardiomyocytes, increased capillarization and an improved left ventricular ejection fraction [31]. The uniformity of such delivery gel for other therapeutic molecules was demonstrated by the slow release of extracellular vesicles [104] and microRNAs [105].

2.2. Nano and micro-particles-based protein delivery systems

Nanoparticles and microparticles can be composed of a wide variety of materials that can be chosen to be inert, non-immunogenic, easy-to-manipulate and biodegradable [106]. Their surface can be functionalized with, for example, antibodies, peptides or small molecules for targeting. Encapsulation and delivery of proteins using nano- and microparticles prevent proteins from being degraded by extracellular metabolizing enzymes, allows sustained release, targets to a specific area by surface ligands, and thereby potentially improving therapeutic efficacy [107,108]. Generally, nano- and microparticles can be quickly degraded due to their large surface area. This higher biodegradability is an advantage compared with hydrogels as this may reduce chronic inflammatory responses within the tissue, provided that the depot lasts long enough for appropriate therapeutic effects. Similarly, as for hydrogels, there is a near unlimited number of materials to choose. Where natural materials may be expected to show better biocompatibility, synthetic polymers, on the other hand, are usually better characterized than natural counterparts and can be tailored due to processing techniques [109,110].

Several proteinaceous nano- and microparticles have been investigated as controlled release formulations for growth factors. Placental growth factor (PlGF) has been shown to stimulate angiogenesis and improve cardiac function [111–114]. In vitro, PlGF-release studies showed a sustained release of PlGF from the nanoparticles over a period of eight weeks. In vivo, increased left ventricular function, vascular density and anti-inflammatory cytokine IL-10 levels, reduced scar formation and proinflammatory cytokines TNF-α and IL-6 were observed in a rat model of ischemic cardiomyopathy [115].

An injectable alginate hydrogel consisted of silk fibroin microspheres, designed for controlled release of IGF-1, was subsequently entrapped into sodium alginate solutions before injection into the rat ischemic heart. The results showed that the continuous release of IGF-1 was enabled by the silk fibroin microspheres, accompanied by reduced infarcted area and enhanced cardiac function [116]. When different proteins were combined within these microcapsules, similar findings were obtained in an ischemic heart mouse model and rat model of chronic heart failure respectively [117,118].

Heparin is a natural polymer with inherent activity as a clinically used anticoagulant drug. It can bind cytokines and growth factors because of the electronegative sulfonic acid and carboxyl domains. Qiao et al. assembled heparin polysaccharide nanoparticle (HepNP) with VEGF for the therapy of myocardial infarction through intravenous injection and found substantially reduced scar formation and improved cardiac function [50].

As an example of synthetic materials, poly (lactic-*co*-glycolic acid) (PLGA)-based nanoparticles are attractive materials due to their biocompatibility and safety profile and regulatory approval [109,119]. Polylactic co-glycolic acid nanoparticles were developed and loaded with VEGF. This approach allowed sustained delivery of VEGF to the targeted site for at least one month in a murine myocardial infarction model, accompanied by reduced infarct size, increased vascular density and left ventricular contractile function, signifying that this nanoparticle-mediated delivery strategy contributed to increased therapeutic potency of VEGF for ischemic heart disease [110]. Chang et al. studied encapsulation of insulin-like growth factor (IGF)-1 in three differently sized particles in a mouse myocardial infarction model (i.e. (60 nm, 200 nm, and 1 μm). The smallest particles showed the best improvement in heart function [51]. Formiga et al. [55] developed PLGA-microparticles, containing VEGF using a double-emulsion evaporation technique in a rat model of ischemia-reperfusion. Localized and sustained protein release was obtained and consequently, a prolonged biological effect of promoting angiogenesis in impaired myocardium was observed. Ultimately a positive remodeling of the heart was seen in animals treated with these VEGF microparticles over one month.

The overproduction of superoxide is known to induce cardiomyocyte apoptosis and plays an adverse role in the development of ischemia/reperfusion injury (IR), resulting in a rapid decline of cardiomyocytes and heart function. Superoxide dismutase (SOD), an endogenous superoxide scavenger, improves cardiac function after myocardial infarction. However, in large animal trials, this approach has failed to show benefits. This may be ascribed to the poor half-life of SOD1 and therefore, prolonged-release may be beneficial. A delivery system based on polyketal polymer was tested in a rat model of IR. Excess superoxide generated in the border zones of the ischemic heart at three days after IR was dramatically reduced by the polymer-encapsulated SOD, while free SOD1 or empty particles failed to do so [120].

An additional benefit of encapsulation can be to enforce proteins with different properties and kinetics to behave similarly. Fibroblast growth factor (FGF1) and neuregulin-1 (NRG1) are growth factors that are involved in cardiac development and regeneration. In clinical trials, they have failed to give therapeutic benefits mainly due to rapid degradation. Pascual-Gil et, al. combined these factors into PLGA microparticles and PEG-PLGA microparticles. Intra-myocardial injection of NRG1 or FGF1-encapsulated PLGA and PEG-PLGA microparticles induced similar improvements in the ejection fraction and angiogenesis after delivered into the infarcted area in a rat myocardial infarction model [109]. No difference was noted between PEGylated carriers vs. non-PEGylated carriers, and equal phagocytosis in the heart was noted. The protective role of PEG against phagocytosis, in this formulation was apparently absent. Two additional studies with this system demonstrated similar findings in a porcine model of ischemia-reperfusion and rat myocardial infarction model [56,121].

Apart from spherical architectures, the carriers can also have different structures. Nanofibers are polymeric materials with a network composed of engineered fibers with diameters $<$ 500 nm. Protein delivery systems based on nanofibers is a relatively new field. Self-assembling peptides can rapidly form steady nanofibers when exposed to physiological pH and osmolarity. Lin et al. injected VEGF combined with self-assembling peptide nanofibers into damaged myocardium and exhibited that VEGF delivery was prolonged within the myocardium for up to two weeks with notably improved angiogenesis and cardiac function in a rat myocardial infarction model [52]. Controlled delivery of PDGF-BB injection with self-assembling peptide nanofibrous gels, targeted to the damaged myocardium for up to two weeks, exhibited inhibition of cardiomyocyte apoptosis, a reduction in infarct size, and improving systolic myocardial function after ischemia/reperfusion in rats [122]. Approaches for cardiac repair include implantation of cells, while these strategies have been reported to be impaired by poor cell engraftment and survival. The sustained myocardial delivery of IGF-1 with self-assembling peptides combined with cardiomyocytes to rat myocardium for 28 days proved to augmented implanted cell survival and myocyte cross-sectional area, improved systolic function after myocardial infarction operation [123]. Moreover, the simultaneous treatment of IGF-1-tethered self-assembling peptide nanofibers and cardiac progenitor cells in the border zone of infarction can promote cardiomyocyte survival and vessel formation, reduce infarct size in a rat myocardial infarction model, although cardiac performance could be only partially improved [124]. Nevertheless, in some cases, encapsulation in microparticles with growth factors fail to alleviate secondary operation risks despite successful angiogenesis with low systemic effects in patients undergoing bypass surgery [125].

SDF-1 is a well-characterized chemokine and appears to be a potent candidate for stimulating regeneration. However, SDF-1 can be easily eliminated by exopeptidases and matrix metalloproteinase-2. Segers et al. employed an improved version of the chemokine, namely S-SDF-1, which is resistant to matrix metalloproteinase-2 and exopeptidase elimination while it retains chemotactic bioactivity and produces less neurotoxin. This was purified and tethered to RAD16-II self-assembling peptides to form nanofibers and was observed to enhance the capillary density and cardiac function 28 days after administration in a rat myocardial infarction model. These findings manifest that driving chemotaxis by local chemokine delivery may be a potential strategy for regeneration [126]. Inspired by the above findings, Kim et al. combined PDGF with FGF-2 in the same hydrogels and observed similar positive results in the same animal model [127].

Nanoparticles can also be injected intravenously. Liposomes are clinically the most advanced intravenous delivery system with characteristics that can be tailored to support the needs of the active ingredient. Wang et al. incorporated VEGF into liposomes, decorated with anti-P-selectin, to target the infarcted rat heart. They found a marked enhancement in angiogenesis and cardiac performance [66]. In a similar approach, Yamada et al. employed Sialyl Lewis X (SLX) ligands to target liposomes loaded with EPO in a rabbit MI model. A striking reduction in MI size and improved cardiac remodeling and function were observed in the EPO-encapsulated liposomes equipped with SLX moiety when compared to controls [67]. Despite the targeting ligands, intravenously administered liposomes delivered their payload to multiple organs after injections, which may hamper their utility for certain applications [128]. Liposomes can also be part of a locally injected system to release their payload gradually. Oh et al. demonstrated the efficacy of a Capryol 90-based gel with VEGF-liposomes encapsulated in a Pluronic F-127 shell on the regeneration of the ischemic heart in rats. This combined system was shown to prolong VEGF delivery and improve neovascularization and cardiac function [49].

2.3. Polymer scaffolds and cardiac patches-based protein delivery systems

2.3.1. Polymer scaffolds

The cardiac application of materials requires that they should be able to resist the cardiac contractile activity [129]. This is especially true for scaffolds, which are tridimensional matrices that integrate with host cardiac tissue, to promote its pumping function. Scaffolds require elasticity and mechanical strength to cope with the dynamic features of the heart. They can be pre-loaded with stem cells to jumpstart the regenerative process instead of solely providing an environment that attracts ingrowth of cardiac cells [130]. Synthetic polymeric scaffolds can be engineered to meet the unique physical requirements and can be equipped with single or multiple cytokines or growth factors to improve cardiac integration [131]. Often a gradual degradation is preferred where the initial physical support provided by the scaffold is followed by controlled and sustained release of bioactive factors to induce tissue formation and scaffold degradation [132–134]. The critical point for the successful application of cardiac tissue scaffolds lies in its capability of promoting vascularization to enhance regionally (stem) cell survival and support the nearby ischemic tissue [135,136]. Delivery of VEGF from a biopolymer augments vessel ingrowth into the scaffolds in a rat model of ischemia, although the long-term effect of angiogenesis within scaffolds after the VEGF source is exhausted still has not been widely investigated [136].

SDF-1α has been widely accepted as a vital regulator in angiogenic processes after myocardial ischemia and the receptor of SDF-1α, CXC chemokine receptor 4 (CXCR4), has been shown as a potent factor for stimulating angiogenesis following MI [137]. The cardiac performance was studied after myocardial infarction following treatment with a polyurethane scaffold loaded with SDF-1α-transfected cells. Two weeks after rats myocardial infarction, sowed scaffolds were embedded epicardially or the cells were infused into the myocardial. Systolic function was remarkably improved in both groups without significant differences between both treatment groups, which would indicate that the scaffolding strategy is not always beneficial compared to simple local injection. Interestingly, a reduced infarction size in two SDF-1α groups was found, while improved angiogenesis was not observed, suggesting that the mechanism of action may not be as anticipated [138].Miyagi et al. injected hydrogels combined with bone marrow-derived mesenchymal stem cells and angiogenic cytokines SCF and SDF-1a in rats myocardial infarction border zone, which was shown to accelerate angiogenesis of the ischemic myocardium and mitigated cardiac injury [60].

Apart from the vascularization, the inflammatory response that follows ischemia-reperfusion can also be a target to improve cardiac function in the long term. The lack of oxygen can lead to a sizable loss of cardiomyocytes, infiltration of inflammatory cells and release of inflammatory cytokines and chemokines [139–142]. Attracted by cell debris and inflammatory response signals, neutrophils infiltrate the infarct region within the first hours after the initiation of ischemia. They bring large quantities of reactive oxygen species and secrete granule components like myeloperoxidase and proteases, thereby aggravating regional vascular and tissue damage [143]. To offset the adverse effects of inflammation, scaffolds can be specifically designed to incorporate anti-inflammatory factors. Holladay et al. successfully developed a collagen scaffold engineered with a plasmid capable of carrying the gene-specific for the anti-inflammatory IL-10, as an efficient strategy to deliver mesenchymal stem cells to the ischemic myocardium of rats. Four weeks post-transplant, the scaffold promoted cell retention and survival, diminished inflammatory responses, as well as improved cardiac function [61].

Myocardial infarction leads to the replacement of myocardium with the formation of a nonfunctional scar, which represents one of the main reasons for the ultimate mortality in MI. The synthetic cardiac scaffold may ameliorate myocardial remodeling and boost myocardial restoration. To this end, poly-L-lactide scaffold (PLLA) was employed for granulocyte colony-stimulating factor (GCSF) release. This glycoprotein stimulates the bone marrow to generate and release granulocytes and stem cells into the circulation. In this study, PLLA-based scaffolds were found to be effectively incorporated into infarcted myocardium in rabbits without triggering a severe inflammatory response. Compared with the non-functionalized scaffold, the GCSF improved vessel density and extracellular matrix composition [144].

2.3.2. Polymeric cardiac patches

In contrast to scaffolds that become an integrated part of the cardiac tissue, heart patches are developed to adhere to the surface of the injured area of the heart after MI. Although not embedded within the tissue, patches still need biocompatible and biodegradable characteristics and offer a mechanical structure that sustains the dynamics of the beating heart.

Marsano et al. implanted the poly (glycerol sebacate) patches with VEGF into mice one week after induction of MI. Four weeks after implantation, VEGF-containing patches were found to dramatically boost survival and differentiation of cardiomyocytes and cardiac function when compared with those without VEGF. The controlled release of VEGF also regulates the formation of complete vascular networks [59], and similar findings were observed in other studies in the ischemic heart of rats [58,62], indicating that cardiac patches combined with growth factors could be a relatively straightforward procedure for cardiac repair after MI.

Although cardiac patches have great potential as a new therapy for cardiovascular diseases [145–149], the lack of degradable polymeric patches on the market suggests that improvements can still be envisioned. In addition, many cardiac patches are developed for epicardial delivery [129,150,151] and need open-chest surgery to be placed. In addition, they present a risk of forming pericardial adhesions that may lead to cardiac dysfunction and increase morbidity and mortality [152].

2.4. Osmotic pump-based protein delivery systems

Using a difference in osmotic pressure across a semi-permeable membrane, one can promote the net movement of water as a driving force for controlled delivery systems. The osmotic pump is easy to develop and operate and can have sustained release profiles [153]. Hermans et al. [154] combined pericardial catheters with a subcutaneously-implanted osmotic minipump in rats to explore whether intrapericardial delivery is functional. They found that this strategy maintains therapeutic levels in the pericardial space for prolonged periods. Landau et al. [155] infused bFGF into the intrapericardial space in a rabbit model of chronic ischemia. Osmotic pump-delivered bFGF indeed promoted new epicardial vessel growth.

3. Delivery systems for DNA

3.1. Naked plasmid DNA-based gene delivery

The most straightforward non-viral gene delivery system applied in vivo is the direct delivery of naked plasmid DNA. Plasmids can be produced by bacteria in large amounts, making it a cost-efficient process with relatively low immunogenic activity and low toxicity. However, the expression levels of the encoded protein after naked DNA injection remain at a low level because of the inefficient entry of the target cell's nucleus. Still, the direct intra-myocardial injection with naked DNA was applied to achieve target gene delivery to the heart in the clinical study [68]. This approach is straightforward and simple. A phase I study, applying plasmid DNA injection for myocardial angiogenesis in patients with coronary artery disease, demonstrated that direct intra-myocardial injection of plasmid DNA encoding human vascular endothelial growth factor VEGF was safe and improved MI symptoms [68].

Gene delivery of VEGF through transient cardiac overexpression by adenovirus delivery or naked plasmid to promote angiogenesis has been successful both in animal models and in patients with myocardial ischemia [156–159]. Hao et al. compared the efficacy and adverse effects of the two approaches in a rat myocardial infarction model and found that both treatments can augment left ventricular function to a similar degree. However, adenoviral gene delivery induced a higher number of apoptotic cells than plasmid and showed increased side effects with no distinct benefit in efficacy over plasmid. These findings imply that plasmid-encoded VEGF might be a better candidate to induce therapeutic angiogenesis in the heart than adenovirus [69]. This probably due to the strong immunogenicity of adenovirus. It will interesting to see if Cardiac specific AAV vectors with relatively low immunogenicity, such as AAV8, AAV9 are better comparing with plasmids.

In patients with severe ischemic heart disease, high-dose plasmid-mediated VEGF gene transfer seemed to be safe. An intra-myocardial injection with a total dose of 3.8 mg plasmid encoding VEGF into the ischemic region using an injection catheter provoked no serious adverse events associated with either VEGF-expression or the injection procedure over the 2-year follow-up [160,161]. Still, the study lacked control subjects, was open-label, and treatment was short-term. Therefore, in a follow-up, in the Euroinject One phase II trial, 80 patients with severe stable ischemic heart disease were divided into two groups: 1. treated with 0.5 mg of plasmid-encoding VEGF, and 2. receiving 'empty' plasmid into the affected myocardial region. Compared with placebo, VEGF gene transfer failed to improve myocardial perfusion, still local wall motion disturbances were remarkably ameliorated three months later. Although no notable treatment effect was found in myocardial perfusion abnormalities, which may occur partly due to the low dose, it denotes a tendency for favorable therapeutic efficacy. These findings underline that further studies are needed to elucidate the potential value of plasmid VEGF gene therapy [162].

In addition to direct myocardial injection, catheter-based intracoronary VEGF gene delivery can be an alternative administration route. Unfortunately, only a few studies are available that describe regional VEGF gene delivery during angioplasty and stenting. Two percutaneous catheter-based delivery of naked plasmid DNA, encoding for VEGF, to the myocardium in patients with myocardial ischemia also showed similar and positive results [70,71].

3.2. Nanoparticles-based DNA delivery systems

Instead of naked plasmid delivery, nanoparticles may have benefits for gene transfer [163–166]. For example, synthesis and purification of nanoparticles are generally faster and more straightforward than the viral delivery system [167,168]. Also, polymeric nanoparticles with encapsulated plasmid DNA can improve target gene expression by improving uptake and protection of the DNA [169]. Also, the encapsulation of DNA in biodegradable polymer particles provides opportunities to control DNA release [170].

One of the most widely used polymers that have EMA approval in several applications is poly (lactide-*co*-glycolide) (PLGA). PLGA shows attractive biocompatibility, biodegradability, and sustained-release properties. PLGA particles loaded with plasmid VEGF injected into ischemic rabbit myocardium improved cardiac function and increased the number of capillaries in the tissue [38]. Still the lack of efficient encapsulation due to the absence of cationic charge makes this approach challenging.

A popular charged polymer that has shown good transfection properties is poly (ethylene imine) PEI. It has an extremely high charge density, which is favorable for plasmid encapsulation yet has drawbacks in cell specificity as it tends to bind to any negatively charged cell membrane.

To overcome this, a reduced charge density copolymer was made consisting of chitosan-graft-PEI-eprosartan. The chitosan was grafted with PEI obtaining a moderately charged polymer. In addition, the polymer is equipped with the drug eprosartan that binds to the angiotensin II type 1 receptor that is overexpressed on cardiomyocytes. In vivo studies in a rat model of MI in which the polymer was loaded with plasmid VEGF and injected intramyocardially, the formulation improved heart function with a significant increase in ejection fraction after local injection in the myocardium. Also, at the cellular level, reduced apoptosis and increased number of capillaries in the infarct and peri-infarct area was seen [171].

Similarly, the high charge density poly-L-lysine (PLL) polymer has been one of the first cationic polymers used for non-viral gene delivery. Compared to PEI it has the important advantage that it is biodegradable, yet it also faces the same issues with cationic charge. A gene delivery vehicle named TerplexDNA was designed which consists of stearyl-modified PLL, low-density lipoprotein (LDL) and plasmid DNA that limits charge density by interaction with the lipids. Transfection efficiency of TerplexDNA after local injection into the myocardium in rabbits was several dozen-fold higher than that of naked DNA. This might be attributed to interaction with the LDL receptor on the surface of cardiomyocytes [172]. Positive therapeutic outcomes in this rabbit model featured reduced cardiac dilation and improved myocardial function after MI [72].

A variety of studies demonstrated positive therapeutic outcomes in animal models after local administration of such targeted cationic polymer systems with reduced charge density. Several avenues have been followed to improve the applicability of these systems further: reduce the invasiveness of the procedure, improve expression and performing the procedure ex vivo.

The invasiveness of intra-myocardial injection can be improved by using an intravenously administered delivery system. Using the phage-identified homing peptide CSTSMLKAC, primary cardiomyocytes in the infarct area could be targeted [173]. Cystamine bisacrylamide-diamino hexane polymers equipped with the homing peptide and D-9-arginine enabled the delivery to, accumulation in, and transfection of the ischemic myocardium after intravenous administration [174].

In order to improve transfection efficiency, engineering of the plasmid with regulatory elements has been investigated. A hypoxia-inducible VEGF gene therapy system, with a combination of erythropoietin enhancer and water-soluble lipopolymer to increase the VEGF expression and enhance the proliferation of the endothelial cells, has been devised showing improved results compared to the system that lacked the engineering [175].

In an approach, focusing specifically on the apoptotic cell death of cardiomyocytes, positive regulators of death receptor-mediated apoptotic signaling pathways have been studied. Src homology region 2 domain-containing tyrosine phosphatase-1 (SHP1) is one of the primary apoptosis-regulatory factors [176]. Kim et al., combined siRNA targeting SHP-1 (siSHP-1) and hypoxia-inducible VEGF expression vector with a stable nanocomplex by using deoxycholic acid-modified PEI. Local injection into the ischemic myocardium of rats resulted in reduced cardiomyocyte apoptosis and infarct size, and increased cardiac vessel formation for the dual-mode carrier compared to each of the single-mode delivery strategies, indicating simultaneous modulation of apoptotic gene silencing and angiogenic gene expression may be an attractive approach in ischemic heart diseases [39].

A strategy to circumvent the difficulties associated with in vivo gene delivery, especially given the inherent cellular toxicity of many delivery systems is to move the procedure ex vivo. Zhu et al. devised a mesoporous organosilica nanoparticle composed of a silica core coated with PEI carrying a plasmid encoding hepatocyte growth factor (HGF). This system was used to transfect bone marrow-derived mesenchymal stem cells ex vivo. After the recovery of the cells, they were applied locally to a rat model of myocardial infarction. Reduced infarct size and interstitial fibrosis, improved vascular density and heart performance were observed, suggesting that this new combined approach of stem cells and ex vivo transfection is sufficient [37]. Still the daunting logistics of such cell-based products may be a significant limitation.

3.2.1. Lipid-based DNA delivery systems

In 1980, Fraley et al. demonstrated the feasibility of delivery of plasmid DNA to monkey kidney cells with lipid-based nanoparticles [177], and Schaefer-Ridder et al. also demonstrated the successful gene delivery through lipid-based nanoparticles [178]. Currently, lipid-based gene delivery systems have been utilized in some clinical trials designed to express therapeutic proteins for the treatment of tumors through various administration routes. In 2001, Xu et al. used a new cationic immunolipoplex system which demonstrated a desirable gene delivery efficiency and anti-tumor efficacy in vivo for systemic p53 gene therapy for cancer [179].

In a randomized, placebo-controlled, double-blind phase II study, patients with coronary heart disease were recruited and received gene transfer with a perfusion-infusion catheter. 37 patients applied with VEGF adenovirus, 28 patients acquired VEGF plasmid lipidic carriers and 38 control patients received Ringer's lactate. After six months of the follow-up period, although intracoronary gene transfer proved to be safe, no significant difference in minimal lumen diameter and proportion of diameter stenosis can be examined among all the study groups [157]. Given the applications of lipid-based gene delivery systems in other fields, it is expected that these carriers will gain importance in the cardiac filed as well, although tissue-specific adaptations may be needed.

One opportunity may be the ex vivo transfection of cells. Mesenchymal stem cells (MSCs) have been used as carriers, overexpressing growth factors, to boost their cardiac regenerative potential [180–183]. Locatelli et al. employed a combination of two lipid-based reagents to optimize plasmid-mediated transfection of ovine MSCs, which exhibited high transfection efficiency and cell viability [184], suggesting that this strategy may help deliver regenerative factors efficiently and transiently in the myocardium.

3.3. UTMD-based DNA delivery systems

Apart from designing chemical constructs that can deliver plasmids, physical techniques that temporarily disrupt cell membrane integrity has also been explored. Currently, a number of studies have investigated using ultrasound-targeted microbubble destruction (UTMD) for nucleic acid delivery [185]. After a plasmid-coated microbubble is administered, the plasmid will be released to the targeted area by using precise ultrasound-regulated microbubble destruction. In addition to the local release, the high energy cavitation of microbubbles contributes to increasing permeability of cell membranes and accelerating the absorption of the gene into the targeted site. The ultrasound-targeted microbubble destruction (UTMD) approach is recognized as a clinically applicable, repeatable alternative and has been utilized for gene and drug delivery in many preclinical models of disease, including cardiovascular diseases [186–189]. Microbubbles have been used in the clinic as the echocardiographic contrast agent, but it hasn't been tailored for delivering purpose. In order to improve plasmid transfection with UTMD, cationic microbubbles with a higher DNA-binding capacity has been tested.

Kobulnik et al. used UTMD to deliver the VEGF plasmid and compared with direct intramuscular injection in a hind-limb ischemia model of rats. In both groups, increases in microvascular blood volume and microvascular blood flow were observed, while microvascular blood flow appeared to be more enhanced in the UTMD group. This may by attributed to a further diffusion and distribution of the transgene that is favorable for improving angiogenesis [75].

In addition to VEGF, a variety of other genes has been tested, like RAC-alpha serine/threonine-protein kinase (AKT). When this gene was UTMD-delivered to ischemic myocardium, reduced apoptosis, increased vascular density and decreased infarct size was observed, underlining the applicability of the UTMD approach for restoration of ischemic rat myocardium [77].

Growth differentiation factor 11 (GDF11), a member of the transforming growth factor-beta family, augments stem cell viability. Du et al. combined GDF11 gene-encoding plasmids on cationic microbubbles with UTMD delivering them to the ischemic myocardium of mouse, and found that this can dramatically enhance GDF11 expression, recruitment of endothelial progenitor cells, angiogenesis and cardiac performance, with decreased infarct size in ischemic hearts [79].

Applying intra-myocardial injection of the plasmid-encoded HGF gene loaded on microbubbles combined with UTMD, significantly increased gene expression in dogs following myocardial infarction [81]. This finding was consistent with Zhou et al. exhibited in a rat myocardial infarction model [82], showing that UTMD-induced gene delivery not only increased the expression of the HGF gene but also improved angiogenesis and fibrosis in myocardial ischemia with a minimal impact on cell viability.

In 2009, Fujii et al. showed that UTMD is helpful to transfect two genes, VEGF and stem cell factor (SCF), simultaneously successfully into the mouse ischemic myocardium. A combination of these two factors led to a remarkably improved vascular density and myocardial blood flow perfusion [74]. They also examined the safety and efficacy for repeated UTMD-modulated delivery of a gene, i.e., stromal cell-derived factor 1(SDF-1), into rat hearts after myocardial infarction. The repeated treatment achieved an evident therapeutic effect with increased vessel density and cardiac function and reduced infarct size [80].

Recently, researchers exploited a targeted microbubble with an anti-intercellular adhesion molecule-1 antibody to specifically target the ischemic vascular endothelium to deliver the angiopoietin-1 gene into the infarct area in a rabbit model. Compared with the non-targeted microbubble group, the targeted UTMD angiopoietin-1 gene treatment group revealed a significantly improved cardiac function, myocardial perfusion and microvascular density in the ischemic region [78].

As an alternative to the delivery of growth factors that stimulate regeneration, inhibitors of adverse remodeling have also been proposed. Increased cardiac matrix metalloproteinase (MMP) activity is strongly associated with the development of heart failure. Inhibition of MMP activity with tissue inhibitors of matrix metalloproteinases (TIMPs) is verified to ameliorate cardiac remodeling and boost cardiac function after ischemic injury [190]. Cationic microbubbles loaded with plasmid-encoded TIMP efficiently delivered the gene through UTMD to ischemic rat myocardium or mouse hind limb tumors [77,191]. To improve tissue targeting, an antibody against MMP2 was added to this system to guide the Timp3 gene to the infarct area of the rat heart. This combined therapeutic strategy appears beneficial for myocardial infarct area binding, especially at the scar and border zone region. It also improved gene transfection efficiency, inhibited infarct thinning and dilation and eventually induced cardiac repair and myocardial function restoration after rats MI compared with those without targeted cationic microbubbles. It seems that UTMD therapy with this specific microbubbles combination may be a promising approach to offer an efficient way for the targeted delivery of genes intended cardiac repair after ischemic injury [83].

In short, numerous studies have shown that UTMD is a novel gene delivery system due to its advantages of non-invasiveness, low immunogenicity and repeatability. UTMD-mediated gene delivery technique can pave the way for multi-gene therapy and have a good prospect in cardiovascular applications. The limitation of this strategy is that the blood opsonization and local shear stress of the blood flow may impair the targeting ability of antibody/cationic surface and microbubbles [192].

3.4. Summarizing discussion

DNA-based delivery of growth factors to stimulate vascularization or inflammation inhibitors to limit adverse cardiac remodeling has been achieved. This type of therapeutic intervention is facilitated by the fact that the DNA molecule is very similar between each of the strategies. Irrespective of whether the therapeutic protein is a growth factor or cytokine, the plasmid DNA is essentially the same molecule with the same physicochemical characteristics and stability. The only difference being in the order of the encoding nucleotides. As a result, DNA delivery systems coalesce around similar themes. One of the most critical hurdles for widespread application is the inefficient entry of plasmid DNA into the nucleus where transcription to mRNA needs to occur. To circumvent this, the delivery of mRNA might be an attractive alternative.

4. RNA delivery systems

4.1. RNA therapeutics

Messenger RNA (mRNA)-based therapies are highly promising for the treatment of various diseases by providing functional protein transiently expressed in desired cells without the difficulties of DNA-based constructs [193]. A significant advantage is the intracellular site of activity –the cytoplasm– which circumvents the need to pass the nuclear membrane. About three decades ago, Wolff et al. [194] first demonstrated that direct injection of mRNA could be translated into protein in vivo, which opened up a new era in the clinical translation of mRNA therapeutics. Nevertheless, several advances in nucleotide chemistry, synthesis, scale-up and delivery were required to bring this approach closer to application. Since these innovations have been introduced relatively recently, we will discuss these in more detail.

The template for mRNA consists of five in cis-acting structural elements, from 5′ to 3′ end: (1) the optimized cap structure, (2) the optimized 5′ untranslated region (UTR), (3) the codon-optimized coding sequence, (4) the optimized 3′ UTR and (5) a stretch of repeated adenine nucleotides (polyA tail) [195]. The cap structure and the poly (A) tail are important regulatory determinants in establishing the translational efficiency of the mRNA and stabilizing the mRNA against decay, while the UTR's regulate the half-life and translational efficiency of the mRNA [196]. Conventional mRNA-based therapy had limited value as a therapeutic because of instability and immunogenicity problems. First, conventional mRNA is readily degraded by the omnipresent extra- or intracellular ribonucleases. Furthermore, mRNA can be recognized by various pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) 3 or 7/8, which bind double-stranded and single-stranded RNA, respectively. This can trigger the innate immune system and subsequently result in the expression of pro-inflammatory cytokines and type I interferons [197–200]. A series of modifications to the vectors used to produce mRNA as well as to the synthetic mRNA itself, were needed to improve the translatability and stability of mRNA and reduce its immunostimulatory activity in mammalian cells and in mice [201].

Chemical modification of nucleotides in mRNA can be used to reduce mRNA immunostimulatory activity. i.e., modifying mRNA by incorporating the naturally occurring nucleosides, including pseudouridine (ψ), 2-thiouridine (s2U), 5-methyluridine (m5U), N^1-methylpseudouridine (m1ψ), N6-methyladenosine (m6A), or 5-methylcytidine (m5C), improves this, likely due to changes in the secondary structure of the mRNA and thereby limiting its recognition by the TLRs and nucleases [202]. Recently, Andries et al. [203] showed that the incorporation of N1-methylpseudouridine (m1ψ) in mRNA resulted in innate immune evasion and increased translational capacity in vitro and in vivo [204].

Furthermore, mRNA 5′ and 3′ UTRs are responsible for recruiting RNA-binding proteins and microRNAs, and they can regulate the half-life and translational efficiency of mRNA. The cap structure and the poly A tail give significant stability and translatability to mRNA by inhibiting RNA decapping and improving resistance to enzymatic degradation [205]. Both can be incorporated into RNA during transcription with the inclusion of an anti-reverse cap analog (ARCA) and by encoding an extended region of polyadenylation, but can also be added enzymatically after the in vitro translation. These modifications can significantly improve the properties of mRNA and extend its application range.

4.2. Production of modified mRNA

In vitro transcription (IVT) of RNA with phage RNA polymerases is currently the most efficient method to produce long sequence-specific RNAs. Although there is a broad variety in methods by which mRNA can be produced, the same basic procedure is followed in all in vitro synthesis protocols. In general, in vitro production of RNA is performed by a phage RNA polymerase using a linear DNA template containing a bacteriophage promotor, the optimized UTR's, the codon-optimized sequence and a mix of the different nucleoside triphosphates. In this way, the resulting mRNA resembles a fully processed mature mRNA as it would naturally be present in the cytoplasm of eukaryotic cells.

After mRNA synthesis, contaminants like ions and residual polymerase, need to be removed. There are different purification methods, which have their own advantages and disadvantages. Lithium chloride precipitation is a widely used method to purify RNA in the laboratory. RNA dissolves readily in water because both are highly polar substances. The monovalent cations in the form of salts can combine with negatively charged RNA through electrostatic interactions and precipitate by charge neutralization. Lithium chloride is attractive for the purification of mRNAs because it can efficiently precipitate larger RNA molecules but does not efficiently precipitate DNA, tRNA and other small RNA fragments, proteins, and unincorporated nucleotides following in vitro transcription [206]. Another method of RNA purification commonly used in the laboratory are silica-based columns; this method relies on the binding of RNA to the solid phase in a chaotropic buffer. Silica-based columns are popular as they allow fast extractions and good yields of high-quality nucleic acid [207]. Finally, size-exclusion chromatography (SEC) columns in fast performance liquid chromatography (FPLC) systems, can rapidly purify homogeneous RNA samples. The method allows rapid and straightforward preparation of milligram quantities of pure RNA in a single day [208]. Many of the smaller contaminants that can induce type I interferons (IFNs) and proinflammatory cytokines though PRRs are removed using these techniques [209,210]. However, long RNA contaminants are not. The use of high-performance liquid chromatography (HPLC) purification can remove these contaminants, yielding higher translation, but without induction of IFNs and inflammatory cytokines and no significant induction of genes associated with RNA sensor activation [211]. The most advanced HPLC approach allows the purification of mRNA that can produce up to 1000-fold more protein in primary cells and in vivo, as compared to the same unpurified mRNA [212].

4.3. The advantage of RNA therapeutics for cardiac repair

mRNA performs its function in the cytoplasm and does not require nuclear localization to mediate protein translation, which makes the delivery of mRNA less demanding than for DNA. Also the expression of mRNA is transient, minimizing the potential risks of prolonged protein expression and/or insertion mutagenesis, where the major approaches are growth factor delivery to improve vascularization and inhibition of inflammation. Zangi et al. introduced new blood vessels around ischemic areas by delivering an mRNA encoding human VEGF into infarcted mouse hearts [63]. Ischemic injury leads to the induction of pro-inflammatory cytokines [213–215], followed by a very rapid inflammatory reaction, which occurs within 2–3 days of ischemia injury. This time frame thereby indicates the window of opportunity for intervention in which desirable gene combinations could be delivered to rescue cardiomyocytes [204].

Since mRNA-based strategies have only become available relatively recently, they can benefit from the latest insights into disease-modifying interventions. Promoting cardiac myocyte regeneration to repair the cardiac injury by introducing specific cytokines is a novel emerging therapeutic approach [216], which can be complemented by modulating miRNA-pathways, such as miRNA-15 [217, 218]. There are three endogenous cardiomyogenic pathways: 1. resident stem progenitor cell pool differentiation to form new cardiomyocytes, 2. pre-existing cardiomyocytes re-entry of the cell cycle to substitute lost cells, and 3. reprogramming of fibroblasts to transdifferentiate into cardiomyocytes. These three opportunities for cardiomyocyte regeneration all involve the delicate regulation of gene expression. It has been reported that modified mRNA can be expressed very rapidly in vivo and remains detectable for up to 10 days [63,204]. This fits nicely with the time frame in which therapeutic intervention should occur.

4.4. The challenges of RNA therapeutics in cardiac repair

Even though RNAs act in the cytoplasm, RNA molecules still must pass significant obstacles to reach their target site. Viral vectors can efficiently transduce RNAs into cardiac muscle cells but can suffer from strong immune responses and compromising genomic stability [219–222]. Here, we discuss synthetic non-viral deliver vectors.

4.5. Naked mRNA delivery

The simplest way to deliver mRNA is to inject the naked molecule into the target tissue, i.e., the infarct region. The simplicity of the method has allowed it to progress furthest with clinical trials underway. AZD8601 is a modified mRNA encoding VEGF that is administered in citrate saline buffer (10 mmol/L citrate, 130 mmol/L sodium chloride pH adjusted to approximately 7.5 with sodium hydroxide) [73]. It was shown that the mRNA displayed remarkable stability in the swine heart tissue possible due to the absence of degradative enzymes in the tissue. The drug was shown to be well tolerable and effective, even administered one week after MI in a minipig model. Still, the doses used in minipigs were relatively high (between 1 and 10 mg) and required direct administration into (peri-) infarct area with a total number of intracardial injections of ~20).

4.6. Lipid-based RNA delivery systems

Lipid nanoparticles are the most commonly used nano-systems for the delivery of RNA agents [223,224]. RNA can be efficiently condensed through electrostatic interaction. The cationic charge can additionally promote the cellular uptake of RNA through endocytosis and/or endosomal escape [225]. DOTMA (N-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride) was the first synthetic cationic lipid used to condense and deliver mRNA into cells [226]. A number of derivatives, such as DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane), are widely used for RNA delivery, particularly in vitro [227]. To improve RNA transfection, neutral helper lipids such as DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) can assist in endosomal escape [224,225]. In vivo, such lipid systems are rapidly cleared through the mononuclear phagocytes system in liver and spleen after intravenous injection, which reduces delivery efficiency. Also, the cationic lipids tend to form aggregates with negatively charged blood components causing adverse effects through capillary blockade.

Inclusion of functional lipids such as polyethyleneglycol (PEG)-phospholipid conjugates can reduce this recognition and electrostatic interactions. In addition, the surface of the particles can be decorated with targeting ligands to improve interaction with target cells. Heart-targeted lipid carriers are promising for MI: For example, by the use of anti P-selectin to target infarcted myocardium [65] or a ligand specific to angiotensin II type 1 receptor (AT1) [228] demonstrated that conjugated - liposomes with were able to target the infarcted heart after intravenous injection in vivo specifically. These studies suggest that liposomes with specific modifications may be useful for delivering therapeutic agents with more precision to the infarcted heart.

It is worth mentioning that some commercially available lipid formulation also demonstrated to result in RNA transfection, even in vivo [229]. In 2013, Zangi et al. showed that intra-myocardial injection of formed lipid-complexes with modified mRNA encoding VEGF resulted in reduced infarct size and improved myocardial outcomes in a murine MI model [63]. They observed that VEGF mRNA leads to improved vessel formation due to pulse-like VEGF overexpression. This stimulates the endogenous epicardial progenitor pool in the peri-infarct area better than sustained protein expression.

More recently, studies have focused on replacing cationic lipids with pH-dependent ionizable lipids. They offer the advantage of cationic charge during RNA-complexation at low pH, neutral charge at physiological pH after injection and restoration of charge at low endosomal pH after intracellular uptake for endosomal escape. Hereby, also balancing between transfection and cytotoxicity. In 2019, the FDA approved the first siRNA drug for amyloid transthyretin, which contains the ionizable lipid(6Z,9Z,28Z,31Z)-hepta-triaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3). Since then, a large number of lipid nanoparticles (LNP) originally developed for siRNA delivery can be optimized specifically for mRNA delivery [230]. Thess et al. used MC3 to encapsulate and deliver mRNA encoding erythropoietin by intraperitoneal or intravenous injection, which achieved therapeutically relevant concentrations of erythropoietin from mice to non-human primates [40]. This shows that the same delivery technology can be used for different RNA lengths and underlining sequences and that such differences are far less important for the delivery of RNA than for the delivery of proteins and peptides. Gilleron et al. developed an analytical platform, based on a combination of quantitative light and electron microscopy (EM), to dissect the mechanisms of LNP-mediated siRNA delivery with high resolution [231]. They found that only a small fraction (1–2%) of siRNAs were released from endosomes and release occurred only at a defined stage of endosomal-lysosomal maturation. Important improvements can be made even after intracellular arrival in the target cell.

Turnbull et al. [41] introduced LNP-loaded modified mRNA encoding green fluorescent protein to rat and pig myocardium by direct myocardial injection. They demonstrated that LNPs enabled efficient, rapid and short-term mRNA expression in the heart. Moreover, optimization of ionizable lipids specifically for specific RNA classes and target tissues may significantly improve the delivery features of LNP. Fenton et al. synthesized a series of ionizable lipids based upon alkenyl amino alcohols [232]. One of them, named OF-2, was formulated with cholesterol, DOPE, C14-PEG-2000 and mRNA coding for erythropoietin into LNPs, that exhibits outperformed other ionizable materials in terms of in vivo erythropoietin production across a broad dose-response window.

Biodegradability is a crucial feature of nanoparticles for in vivo applications as the accumulation of non-biodegradable lipids has already been demonstrated to induce adverse effects or even acute mortality in animal models. Ester groups are the most commonly used functional groups for enhancing the biodegradability of biomaterials [233]. Sabnis et al. reported a biodegradable ionizable lipid, named lipid 5, which contains a primary ester-containing lipid tail [234]. They found that the presence of this primary ester led to faster clearance from the liver compared to MC3 in non-human primates. The liver is usually the dominant organ for LNP accumulation [235,236].

Targeting specific organs or tissues beyond the liver with LNPs is more challenging. Recently, Cheng et al. presented an approach called selective organ targeting (SORT), whereby LNPs containing nucleic acid based therapeutics are bioengineered to induce liver-, spleen- and lung-specific gene regulation [237,238]. Unfortunately, a heart-specific formulation was not investigated but it does provide a strategy to arrive there. LNPs have also been equipped with ligands for cardiac tissue-specific receptors [239]. The Wang group designed anti-cTnI (cardiac troponin I) antibody-modified nanoparticles containing antisense against microRNA-1 (AMO-1) to target the ischemic myocardium based on the overexpression of cTnI in this tissue [28].

4.7. Polymer-based RNA delivery systems

Similar to DNA, RNAs have also been delivered with cationic polymers such as PEI, chitosan, PLGA, dendrimers and mixtures thereof. Similar to lipid-based delivery systems, in recent years, the polymers have transitioned from use for siRNA and DNA delivery to mRNA delivery.

Polyethyleneimine (PEI) is one of the most potent and commonly used synthetic cationic polymers [240,241]. To circumvent issues with biodistribution, the polyplexes can be directly injected into the myocardium. Kim et al. constructed a nanoparticle with a low molecular weight PEI to deliver an siRNA against SHP-1 (Src homology 2 domain-containing protein tyrosine phosphatase-1) to the myocardium of rats in an ischemia/reperfusion model, resulting in effectively silenced SHP-1 gene expression in cardiomyocytes, leading to a significant inhibition of cardiomyocyte apoptosis under hypoxia [43]. Huang et al. delivered a synthetic chemically modified mRNA encoding insulin-like growth factor-1 (IGF-1) to the area at risk in an in vivo murine model of myocardial infarction (MI) by direct myocardial injection [44]. Efficient and transient protein expression was measured, promoting cardiomyocyte survival and abrogating cell apoptosis under hypoxia-induced apoptosis conditions.

Transient protein expression induced by mRNA during myocardial infarction strongly influences the cardioprotective role of IGF1. Zangi et al. found that the prolonged activation of IGF-1 signaling pathways in the heart post-MI had negative consequences [242]. Constitutive expression of IGF-1 led to the formation of epicardial adipose tissue (EAT) post-MI. Epicardial fat is a unique fat compartment between the myocardium and the visceral pericardium. Excessive epicardial fat deposition around the heart may result in the development of atherosclerotic plaques in the coronary vessels [243]. Therefore, the transient expression of mRNA may be preferred over prolonged-expression.

4.8. Microbubbles

Similarly, as for DNA delivery, ultrasound-targeted microbubble destruction (UTMD) could be a valuable physical method for the delivery of RNA therapeutics. Recently, many studies have used UTMD for local RNA delivery in the cardiovascular system [52,70]. In 2015, Chen et al. [50] used UTMD to deliver the human thymosin beta 4 (TB4) gene into healthy rat hearts TB4 stimulated differentiation of resident adult cardiac progenitor cells. They demonstrated that cardiac-specific delivery of exogenous human TB4 gene efficiently stimulates proliferation and differentiation of resident adult cardiac progenitor cells into three intact cardiac cell lineages: vascular endothelial cells, coronary artery smooth muscle cells and cardiac muscle cells [50]. Alter et al. also showed that single-stranded oligonucleotides could be delivered locally to the heart via UTMD, inducing the transcription of dystrophin in mice cardiomyocytes [86].

RNA molecules can be precisely delivered to the cardiovascular system under the guidance of ultrasound, which is unique from the synthetic delivery vehicles discussed above. Tsunoda et al. successfully delivered siRNA against GFP into the heart in GFP transgenic mice [244]. However, the siRNA was observed only in cardiac endothelial cells but not in cardiomyocytes. Interestingly, others have shown that labeled miRNAs were targeted to the cardiomyocytes via UTMD [187], associated with a minimal temporal neutrophil invasion due to the treatment. Therefore, UTMD-induced delivery of RNAs is still rather complicated, and many factors can affect its targeted delivery, including the type and dose of microbubbles [86], ultrasound intensity, ultrasound frequency and ultrasound pulsing [245–247]. However, when all of these factors are well balanced, the UTMD-induced delivery of RNAs is an up-and-coming technique for the treatment of cardiovascular disease, including the myocardium [248].

5. Extracellular vesicles mediated cardiac repair

Extracellular vesicles (EV), sometimes also referred to as exosomes, are increasingly recognized as important biological information carrier for intercellular communication. EVs contain proteins, small RNAs, mRNAs, and second messengers some of which have been shown to play important role in cardiac repair as summarized in our recent position paper [249]. At the same time, as listed in Table 1, there are also downsides associated with the use of EVs. EVs carry a high cost for production and upscaling and so far lack efficient cargo loading. Recently, we and others have developed different strategies for biofabrication of cell derived EV-like vesicles with similar biological function as EVs [250–252]. These cell-derived nanovesicles are formed by physical disruption of the cellular membrane. Even though these artificial vesicles have shown some similarity to the EVs and even activate the ERK pathway, a head-to-head comparison with EV still needs to be performed. Inspired by the structural similarity of the lipid bilayer between liposomes and EVs, bottom-up approaches have also been explored to produce fully artificial EV mimics. Using either traditional liposome formation in the presence of exosomal membrane proteins [253] or using synthetic lipids that mimic exosomal lipid composition [254], this bottom-up production method enables large scale clinical grade vesicles. However, given the biological complexity of EVs, it remains to be determined to which extend they can truly mimic natural EVs.

6. Perspective

There is a growing understanding of the biological mediators underlying MI, the lack of myocardial regeneration and the progression to heart failure. The use of biologicals to modulate these disease mediators is exciting. DNA, RNA and proteins all can be used for this activity. However, direct delivery of these biomolecules is not always effective.

Delivery systems can help to keep these biomolecules stable, offer controlled release and help to traverse the barriers to the site of activity. Precisely targeted delivery of vectors helps to reduce toxicity and improve drug availability [255]. At this moment, a broad variety of delivery systems have been tried, from which frontrunners will be selected that combine safety and performance.

For the future, new biological mediators will be discovered and the complex interactions between these mediators, their pharmacokinetic and pharmacodynamic profiles will be revealed. This opens the way for an advanced delivery system that can even better support the biological's needs for optimal therapeutic efficacy.

Acknowledgments

This project is supported by EVICARE (No. 725229) of the European Research Council (ERC) (to J.P.G.S.), ZonMw-TAS Program (No. 116002016, to J.P.G.S. and Z. L), The Research Project is financed by the PPP Allowance made available by Top Sector Life Sciences & Health to Hartstichting to Stimulate Public-private Partnerships (No. 2018B014, to J.P.G.S. and Z. L). EXPERT (No. 825828) of the EU Horizon 2020 Research and Innovation program (to R. S.); TORNADO (No. 16169) NWO High Tech Systems and Materials Grant (to R.S.). J. F and Q. Y are supported financially by CSC fellowship from Chinese Research Council.

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