

The potential for nanotechnology to improve delivery of therapy to the acute ischemic heart

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Abstract

Treatment of acute cardiac ischemia remains an area in which there are opportunities for therapeutic improvement. Despite significant advances, many patients still progress to cardiac hypertrophy and heart failure. Timely reperfusion is critical in rescuing vulnerable ischemic tissue and is directly related to patient outcome, but reperfusion of the ischemic myocardium also contributes to damage. Overproduction of reactive oxygen species, initiation of an inflammatory response, and deregulation of calcium homeostasis all contribute to injury, and difficulties in delivering a sufficient quantity of drug to the affected tissue in a controlled manner is a limitation of current therapies. Nanotechnology may offer significant improvements in this respect. Here, we review recent examples of how nanoparticles can be used to improve delivery to the ischemic myocardium, and suggest some approaches that may lead to improved therapies for acute cardiac ischemia.

Executive summary

Cardiovascular disease is the leading cause of human death in the western world. Although timely reperfusion is essential after coronary occlusion, reperfusion also contributes to injury.

Although progress has been made in treating chronic symptoms of myocardial ischemia, there is still scope for improved treatment in the acute setting.

Reducing injury in the acute setting using antioxidants, calcium channel modulators and anti-inflammatory treatments improves outcomes in experimental models but therapies have not translated well to the clinic.

The ability to treat the myocardium is hampered by poor retention and specificity – problems that could be addressed through the use of nanoparticles.

In general, liposomes, micelles, and polymer and inorganic nanoparticles have been investigated for delivery of antioxidants, calcium channel antagonists, anti-inflammatory, anti-arrhythmic, and other cardioprotective drugs in small animal models.

There are limited examples of the use of ‘smart’ nanomaterials or multifunctional nanoparticles combining imaging/diagnostics with therapy to treat acute cardiac ischemia.

Mitochondrial targeting of drugs remains a relatively unexplored area, although recent results suggest this route may be a promising future direction achievable using nanoparticles.

Nanoparticle-based transfection, in conjunction with small-molecule therapies and imaging, may also offer significant benefits in treating the ischemic myocardium.

Introduction

Cardiovascular disease is the leading cause of death worldwide.[1] In particular, ischemic heart disease represents a significant burden, because although treatment has improved considerably, many patients still progress to chronic hypertrophy and heart failure.[2] The interrupted blood flow during myocardial infarction and depletion of oxygen supply to the metabolically demanding heart results in death of cardiomyocytes, which in turn, leads to cardiac hypertrophy and eventual heart failure. Under these conditions the heart can no longer maintain a sufficient flow of blood to meet the demand for oxygen in the body.

After an ischemic episode, the adage that ‘time is muscle’ reflects the importance of restoring the oxygen supply to affected cardiac tissue to limit damage and preserve function.[3] In acute ischemia and subsequent reperfusion, a number of abrupt biochemical changes compromise cardiomyocytes and the endothelial cells that line the vessels of the heart.[4] Depletion of oxygen decreases oxidative phosphorylation, resulting in a switch to anaerobic glycolysis, a reduction in intracellular pH, and mitochondrial membrane depolarization. Reperfusion initiates overproduction of reactive oxygen species (ROS), and leads to a cascade of events culminating in intracellular Ca^{2+} overload and an inflammatory response.[5] A number of therapies have shown potential to limit damage in experimental models by addressing these pathological mechanisms, but many suffer from problems of limited bioavailability and poor specificity which have brought about their failure in clinical trials.[4, 6]

The advances that have been made already in the treatment of cardiovascular disease are significant, which ‘raises the bar’ for any new technologies in the field.[7] Indeed, such progress has shifted the focus of research from acute treatment towards longer-term consequences such as prevention of heart failure and regeneration of heart muscle.[8] But in respect of unmet challenges in cardiovascular disease, myocardial reperfusion injury remains a therapeutic target for potential improvement.[5] The extent of the initial injury—that is, infarct size [8]—is the principal determinant of adverse post-injury effects, and so it follows that therapies that reduce the extent of injury in the acute setting are critical to post-injury outcome. Reducing the effects of acute cardiac ischemia remains an important goal in cardiovascular research.

Nanotechnology encompasses some novel approaches to diagnosing and treating cardiovascular disease, and offers the benefit of significant improvements in specificity and bioavailability in treating the ischemic myocardium and ameliorating reperfusion injury.[9, 10] The use of nanoscale drug delivery in the heart is gaining interest.[4, 11] While the

applications of nanotechnology in cardiovascular disease are broad and include the design of new contrast agents for detecting infarcted regions and atherosclerotic plaques, scaffolds for repairing infarcted tissue through stem cell implantation, and stent patterning to encourage biocompatibility,[10] in this article we focus on recent progress on how some early-stage therapies might be better directed to the acute ischemic myocardium using nanoparticle-based delivery systems. There remains much to learn about cardiovascular disease and the myocardium, and inventive exploration in this area will undoubtedly offer new insights into both the healthy and pathological conditions of the heart.

We have divided this review into sections based on therapeutic approach. Some therapies target reactive oxygen species and the redox state, others aim to modulate Ca^{2+} flux, and others alleviate damage caused by inflammation. Although there are many other therapeutic strategies for repairing the post-infarct heart, including stimulating angiogenesis, implanting stem cells for therapy, and using scaffold-based tissue engineering approaches, here we will focus on the acute phase of ischemia–reperfusion injury and delivery of therapies to prevent or ameliorate injury rather than repair it. We will conclude with a view to the future and a discussion of some of the benefits of nano-based delivery that we believe may offer exciting potential but have not yet been comprehensively explored.

Targeted delivery improves existing therapies for ischemia–reperfusion injury

A variety of nanoparticle systems exist that have been explored for their potential in delivering therapies to the myocardium. These include dendrimers, polymeric nanoparticles, liposomes, and micelles. First, we will discuss studies that do not deliver therapeutics but constitute important insights into nanoparticle targeting and distribution, and then literature relating to myocardial delivery will be reviewed according to the therapeutic aim.

Studies of targeting and distribution of nanoparticles

There are a variety of studies examining the ability to target nanoparticles to regions of cardiac ischemia. This is useful either for formulations that are to be injected intravenously or for immobilization of nanoparticles within the myocardium itself. Most nanoparticles make use of passive or active targeting strategies, which are well explored in the context of delivery to tumors, but less comprehensively characterized in the myocardium.[12]

Dvir *et al.* showed targeting of liposomes to model myocardial infarction using a ligand specific for angiotensin II type 1 (AT1) receptor, which is overexpressed in infarction.[13]

They confirmed specificity for cardiomyocytes in culture and in a permanent murine left anterior descending (LAD) artery ligation in which liposomes were injected intravenously. By showing that nanoparticles could cross endothelial barriers more easily when disrupted by the consequences of ischemia, and that these liposomes specifically targeted the injured myocardium, they demonstrated the benefits of an active targeting strategy during myocardial infarction (MI) *in vivo*.

Work in Strijker's group demonstrates the specificity with which micelles and liposomes can passively target the myocardium in murine transient and permanent LAD artery occlusion models using intravenous injection of labelled, PEGylated liposomes. In acute transient occlusion (up to 24 h post-MI), micelles and liposomes displayed a highly specific accumulation in infarcted tissue.[14] After 24 h, most particles had extravasated but some liposomes remained in the vasculature. In the chronic injury (assessed 1 or 2 weeks post-MI), only micelles could be found in the infarcted region. Similar results were obtained in permanent occlusion but with somewhat reduced specificity.[15] The authors attributed the reduction in specificity to the formation of scar tissue with limited vasculature in the chronic setting, which restricts access to smaller nanoparticles. Taken together, these results show that although delivery of nanoparticles is difficult during ischemia because blood flow is severely reduced, there is still the potential for extravasation due to endothelial disruption. Also it is clear that the size of nanoparticles used affects extravascular access to the infarct, and this is an important consideration as demonstrated by limited nanoparticle accumulation in chronic injury.[14] The authors suggest that micelles are well-suited to acute MI cardioprotection and treatment because they accumulate in necrotic myofibers, whereas liposomes, which accumulated slower but more persistently, might be better suited to targeting the vasculature and promoting angiogenesis.[15]

Galagudza *et al.* prepared silica nanoparticles modified with annexin V, an endogenous protein. Annexin V can bind to phosphatidylserine in the presence of calcium, but only following injury, when phosphatidylserine is translocated to the outer surface of the cell. Using this particle, they were able to selectively accumulate particles in ischemic/reperfusion injury in rats, while avoiding healthy myocardial tissue.[16]

Rehberg *et al.* compared the behavior of quantum dots bearing either negatively-charged carboxyl groups or positively-charged and PEGylated amine functionalization in a transient murine LAD ligation model. Cationic amine-QDs strongly associated with microvessel walls only in ischemic tissue, whereas anionic carboxyl-QDs associated (more weakly) with both ischemic and non-ischemic tissue.[17]

Sun *et al.* examined the effect of molecular weight on the cardiac distribution and kinetics of PEG in healthy and ischemic murine myocardium.[18] Ischemia induced by isoprenaline had little effect on the pharmacokinetics of both 20 and 40 kDa PEG. The larger of these displayed enhanced cardiac targeting due to the EPR effect caused by ischemia, and the smaller was more rapidly distributed to and cleared from the myocardium.

Dasa *et al.* used phage display to identify peptides specific for various cell types present in the infarct/border zone of reperfused murine ischemic injury, including cardiomyocytes and endothelial cells.[19] One of these peptides was conjugated to liposomes loaded with AZ7379, a PARP-1 inhibitor, to demonstrate cardiomyocyte-specific inhibition of PARP-1. Targeting using these peptides led to improved PARP-1 inhibition in the cell types studied.

Mediators of ischemia–reperfusion injury and associated therapeutic targets

In this section, we discuss some of the possible interventions for acute ischemia–reperfusion injury and the reasons why existing therapies for each of these might fail. We highlight recent examples of nanoparticle-based delivery strategies from the literature (summarized in Table 1). For more detailed information on the pathology of myocardial ischemia–reperfusion injury and alternative therapies, we direct the reader to some recent reviews.[5, 8]

Oxidative stress

Reactive oxygen species (ROS) are formed as a by-product of oxidative phosphorylation when electrons are donated to molecular oxygen (to produce superoxide anion) via the electron transport chain in mitochondria. Superoxide is also produced by a variety of enzymes, including NADPH oxidases, xanthine oxidase, nitric oxide synthases, lipoxygenases, and myeloperoxidase.[20] ROS play roles in redox signalling and defence within cells, but they can also cause damage through lipid peroxidation, DNA adduct formation, and oxidative damage to proteins and cellular machinery. The sudden restoration of oxygen during reperfusion results in a burst of ROS production by mitochondria, leading to oxidative stress, which predisposes the heart to development of ischemia–reperfusion injury, cardiac hypertrophy, and heart failure.[21] Reactive oxygen species and their contributing role in ischemia–reperfusion injury and cardiovascular health in general have been recently reviewed.[20]

There are many endogenous and exogenous radical scavengers that can be used to neutralize ROS or their products.[22] However, small-molecule antioxidants have failed to show

efficacy in clinical trials, due to non-specificity and lack of potency to scavenge free radicals produced in excess at pathological sites when administered systemically. Additionally, there are a range of endogenous cellular antioxidants that maintain redox homeostasis, including scavenging enzymes like superoxide dismutases and glutathione peroxidase, and phospholipid hydroperoxides to neutralize ROS or their products[21], but these are rapidly cleared.[23] In light of these failures, it has been suggested that treating the source of ROS using specific inhibitors may be more effective than use of antioxidants, which are rapidly depleted at sites of greatest ROS generation.[24]

Although ROS scavengers, including small molecules and enzymes, and inhibitors that target ROS-producing proteins (such as NADPH oxidases) are known to be cardioprotective in a range of experimental models, they are either ‘too late, too little, too nonspecific,’ in the case of antioxidants, or associated with possible side effects caused by removal of the signaling and defensive mechanisms of ROS.[24] It has also been suggested that some antioxidants may be unable to enter cells on their own, and that mitochondrial-specific antioxidants may be more effective.[5] Nanotechnology offers a way to deliver relatively larger doses of antioxidants at the time of reperfusion specifically to the myocardium, or to improve delivery of inhibitors that are not sufficiently specific. In this way, antioxidants coupled with other therapies for site-specific delivery may still prove useful, and so previously discarded drugs may be worthy of re-examination.

Somasuntharam *et al.* used polyketal nanoparticles (~500 nm) to deliver siRNA against Nox2 in RAW 264.7 macrophages and mice following LAD coronary artery ligation.[25] Enzymes of the NADPH oxidase (Nox) family are known contributors to production of superoxide in the myocardium, and Nox2 knockdown prevents apoptosis of cardiomyocytes and cardiac remodeling. Nanoparticles delivering siRNA against Nox2 significantly decreased expression of Nox2 mRNA both *in vitro* and *in vivo*, and improved myocardial function 3 days post-injury as measured by absolute change in fractional shortening.

Corvo and coworkers have investigated the possibility of presenting superoxide dismutase (SOD) on the exterior surface of liposomes.[23] SOD enzymes are rapidly cleared from the blood, resulting in low accumulation at sites of ROS overproduction. Surface-modified liposomes present SOD immediately and may represent a useful approach for the simultaneous delivery of antioxidant enzymes with other slower-release therapies at the time of reperfusion. In comparison, rather than presenting SOD on the surface, Howard *et al.* used liposomes functionalized with antibodies against Platelet Endothelial Cell Adhesion Molecule (PECAM-1) and loaded with EUK-134, a SOD/catalase mimetic, to target delivery to the endothelium and reduce inflammation in acute pulmonary injury.[26]

Soumya *et al.* used guar gum nanoparticles (~70–170 nm) to deliver a selenium salt antioxidant in an *in vitro* model of ischemia in H9c2 rat cardiomyoblasts. They claimed that selenium contained within nanoparticles was more effective than either component alone in protecting cells against changes in a variety of antioxidant biomarkers.[27] Also using a selenium-based antioxidant, Kan *et al.* have shown that phenyl-2-aminoethyl-selenide encapsulated in liposomes can protect against cardiac damage induced by doxorubicin.[28] Doxorubicin is an anthracycline anti-cancer drug. Its use is limited by ROS-mediated toxicity because it induces changes similar to cardiac ischemia, including oxidative damage and development of cardiac hypertrophy.[29] Using a similar doxorubicin injury model, Cote *et al.* encapsulated two antioxidants in Pluronic F-127 micelles and demonstrated protection of H9c2 cardiomyocytes against doxorubicin-induced ROS damage.[30] In these studies, nanoparticle-encapsulated drugs were more effective than drug alone.

Johnson *et al.* used G4 PAMAM dendrimers to facilitate the localized release of nitric oxide from *S*-nitroso-*N*-acetylpenicillamine (SNAP) in *ex vivo* rat hearts.[31] Nitric oxide can limit ischemia–reperfusion injury, but can also be detrimental at higher concentrations, so a controlled, localized and highly specific release mechanism is necessary. Perfusion was halted to induce ischemia for 20 min, and dendrimer-modified SNAP was administered at the time of reperfusion. The dendrimers in this study were functionalized with multiple SNAP molecules and regulated the release of nitric oxide through glutathione-triggered liberation.

Simón-Yarza *et al.* prepared PLGA nanoparticles (~150 nm) containing 35% w/w coenzyme Q10 (ubiquinone).[32] The nanoparticles in this study were used to improve oral bioavailability of this antioxidant rather than being targeted to the myocardium, but improved ejection fraction 3 months after LAD coronary artery ligation in Sprague-Dawley rats.

Intracellular calcium overload

Many sources of calcium contribute to pathological Ca^{2+} overload in the heart. Intracellular and mitochondrial Ca^{2+} overload is initiated at the beginning of acute ischemia[5] as a result of interrupted electron transport, and consequent ion exchange activity. The L-type calcium channel is the principal source of calcium entry in cardiac muscle, and increased calcium influx through the channel contributes to altered calcium homeostasis during reperfusion. In addition, calcium-induced calcium release from the sarcoplasmic reticulum contributes to damage during reperfusion when neutral cytoplasmic pH is re-established and the mitochondrial membrane becomes hyperpolarized, driving Ca^{2+} into mitochondria and opening the mitochondrial permeability transition pore (mPTP).[5, 33] Calcium overload has

a negative effect on the contractility of the myocardium, and mPTP formation is linked to cell death by necrotic and apoptotic mechanisms.[34] Also, there are well-established connections between intracellular calcium and ROS concentrations, and subsequent myocardial dysfunction, hypertrophy, and remodeling.[2] Calcium mishandling and overproduction of ROS can activate Ca²⁺-calmodulin-dependent protein kinase II (CaMKII), which contributes to arrhythmogenesis through ryanodine receptor stimulation (RyR) and increased sarcoplasmic reticulum Ca²⁺ leak, triggering spontaneous action potentials.[35] Accordingly, inhibiting Ca²⁺ flux reduces oxidative stress, arrhythmia, and reperfusion injury in animal models and in the clinic.[2]

The involvement of mitochondria in the mishandling of Ca²⁺ during injury means the use of mitochondrial therapies such as antioxidants, sirtuin stimulators, K⁺ channel activators (which regulate mitochondrial membrane potential) and mPTP inhibitors can be beneficial in reducing ischemia–reperfusion damage.[33, 36] For example, Cyclosporine A reduces mPTP opening and is cardioprotective when administered during reperfusion both *in vitro* and *in vivo*. [36, 37] Targeting to mitochondria has previously been achieved through cationic conjugation or through the use of mitochondrial-targeting peptides, but this strategy is drug-specific. Additionally, while some drugs can act directly within mitochondria, most therapies would benefit from improved targeting to improve efficacy and reduce dosages and side-effects.[33]

Although the effect of Ca²⁺ flux in the development of hypertrophy is well recognized, and antagonists of the sarcolemmal Ca²⁺ channel and mitochondrial Ca²⁺ uniporter can improve outcomes after ischemic injury, negative inotropic effects and drug–drug interactions have resulted in the failure of calcium channel antagonists in a number of clinical trials.[2, 5] L-type Ca²⁺ channel antagonists lack specificity and demonstrate side effects because they fail to discriminate between cardiac and vascular smooth muscle.[2] Ca²⁺-modulating strategies, including calcium channel and mPTP inhibitors and antiarrhythmic drugs, can also be delivered using nanoparticles. The principal benefits lie in improved targeting, either to mitochondria, or specifically to cardiac calcium channels.

Work in our own group has shown that a peptide that inhibits the L-type calcium channel can be delivered using polymer nanoparticles ~200 nm in size, and may constitute an effective route for inhibiting ischemia–reperfusion injury.[38] Interestingly, we have found the use of a peptide directed against the alpha-interacting domain (AID) of the L-type calcium channel to be highly specific for cardiac muscle.[39] AID peptide conjugated to a TAT internalization sequence was administered at the time of reperfusion in an acute IR model after transient ligation of the coronary artery in rats. The AID peptide had no effect on blood pressure. This

is surprising, because in accordance with the similarity of cardiac and vascular smooth muscle L-type calcium channel variants, both cell types have similar alpha interacting domains but it is consistent with the fact that the peptide has very little effect on calcium influx and does not induce negative inotropic responses.[52] The efficacy of the AID peptide was preserved when delivered using polymer nanoparticles, but a more extensive distribution of nanoparticles throughout the myocardium was evident, which may facilitate greater dissemination of therapeutics to vulnerable ischemic tissue prior to reperfusion (Figure 1).[38, 40] The nanoparticles were imaged in cardiomyocytes using rhodamine fluorescence and in the myocardium using magnetic resonance imaging (MRI). Nanoparticles were also used to facilitate the simultaneous delivery of peptide with small-molecule, poorly-soluble antioxidants curcumin and resveratrol.[40] This kind of multifunctional approach, which aims to target both uncontrolled Ca²⁺ flux and ROS-induced damage while also facilitating multiple imaging modalities, draws on one of the principal strengths of nanoparticle-based therapies.

Egashira's group have reported that cyclosporine A (CsA), an inhibitor of the mPTP, could be delivered to mitochondria using PLGA nanoparticles.[37] Using models of ischemia–reperfusion injury in cultured rat cardiomyocytes and in mice *in vivo*, greater mitochondrial targeting of nanoparticles was observed following injury. CsA delivered using nanoparticles reduced left ventricular remodeling and fractional shortening 4 weeks post-injury, and showed improved cardioprotection against ischemia–reperfusion injury. CsA can act on other targets within cells,[33] so nanoparticle-mediated targeting of CsA to mitochondria may improve cardioprotection through more specific delivery of mPTP inhibitors.

Amiodarone is a class III anti-arrhythmic drug, which despite being highly effective, also produces negative hemodynamic effects. Amiodarone acts on a number of ion channels but like most anti-arrhythmic drugs, fails to discriminate diseased tissue, and could be improved using targeted delivery.[41] Barle *et al.* improved the solubility of amiodarone and reduced its acute toxic effects by preparing a nano suspension of the drug, enabling a higher dose without also increasing adverse hemodynamic effects,[42] and Takahama *et al.* have shown that targeted delivery of amiodarone using PEGylated liposomes reduces arrhythmia after brief ischemia–reperfusion in rats.[43] In this study, intravenous injection of liposomal amiodarone resulted in lower mortality, shorter duration of lethal arrhythmia, and fewer hemodynamic side effects than free amiodarone.

Recent work in a model of heart failure has shown that decorating biodegradable polyketal nanoparticles with *N*-acetylglucosamine increased nanoparticle uptake in isolated cardiomyocytes and successfully delivered a calcium-binding protein, S100A1.[44] S100A1

cannot cross the cell membrane alone, and the combinatorial effect of delivery using nanoparticles targeted with *N*-acetylglucosamine to both promote cardiomyocyte uptake and modulate calcium-regulating proteins is particularly important in this case. Although the effect of S100A1 alone is controversial, and the effectiveness of this strategy remains to be confirmed in an *in vivo* model, the potential advantages of this nanoparticle-based combination therapy are clearly demonstrated. We expect that a similar approach in treating ischemia–reperfusion would be an exciting development.

We also note the use of nanoparticles to assist with oral bioavailability. Diltiazem hydrochloride, a non-dihydropyridine calcium channel inhibitor, has been encapsulated with high efficiency in sodium alginate nanoparticles (400–475 nm mean diameter) cross-linked with tannic acid.[45] Nitrendipine, a dihydropyridine calcium channel blocker with poor oral bioavailability, has been formulated in a solid-lipid nanoparticle preparation.[46]

Inflammation plays both beneficial and detrimental roles in ischemia–reperfusion.[5, 47] While chemokine signaling and infiltration of neutrophils assists in clearing necrotic cardiomyocytes and cellular debris,[19] inflammation can also contribute to the ‘no-reflow’ phenomenon and failed resolution of inflammation leads to heart failure.[48] Chemokine signaling begins with hypoxia in the microvasculature in the hours following ischemia,[3] and administration of anti-inflammatory drugs during reperfusion appears to be beneficial in experimental models but so far has yielded mostly negative results in the clinic.[3, 5] It is interesting to note that many anti-thrombotic and thrombolytic drugs also exhibit an inflammation-modulating effect.[49] The role of inflammation in acute ischemia–reperfusion injury is therefore becoming a topic of considerable interest.[50] Since inflammation has the potential to prevent complete reperfusion in the acute ischemic setting, and is linked with progression to heart failure, simultaneous therapies that mitigate the inflammatory response while also targeting other aspects of ischemia–reperfusion pathology could be advantageous. Furthermore, the treatment of cells with nanoparticle-encapsulated anti-inflammatory drugs may improve transfection for gene therapy that could be used for later myocardial repair.[51]

Recent work shows that macrophage polarization is an important consideration in myocardial injury. An adenosine 2B receptor agonist[52], cellular postconditioning [47], and silencing of interferon regulatory factor 5 in macrophages[53] have all been linked to a shift away from a proinflammatory M1-like macrophage phenotype in models of myocardial ischemia with a corresponding improvement in injury outcome. In particular, in this area, lipid nanoparticles were used to protect siRNA against rapid degradation, facilitating its delivery to silence

expression of transcription factors regulating pro-inflammatory cytokines such as TNF- α and IL-1 β ,[53] and the chemokine receptor CCR2 in inflammatory monocytes.[54] To date, relatively little work has made use of nanoparticles to deliver drugs that modulate macrophage phenotype.

Nadig *et al.* tested the ability of micelles to deliver the immunosuppressive drug rapamycin to endothelial cells, assessing IL-6 and IL-8 as markers of inflammation.[55] They functionalized micelles with cRGD peptide in order to target micelles to $\alpha_v\beta_3$ integrins located on the surface of endothelial cells. Using *in vitro* models and aortic grafts of human endothelial cells, cRGD targeting increased micelle internalization by more than 50%.

Nagaoka *et al.* recognized deficiencies in drug targeting in administering statins at the time of reperfusion, and used PLGA nanoparticles to deliver pitavastatin in rats undergoing a transient occlusion of the LAD coronary artery. Therapies were administered at the time of reperfusion. Nanoparticles containing pitavastatin reduced acute MI size, cardiomyocyte death and monocyte-mediated inflammation, and also reduced chronic left ventricular dysfunction, fibrosis and hypertrophy in the infarct border. In contrast, the free drug, even at the maximum soluble dose, did not result in these same outcomes. Interestingly, treatment had an anti-inflammatory effect that was independent of mPTP opening. The authors successfully completed a phase I clinical trial (UMIN 000014940) to investigate the safety of a single intravenous infusion of this nanoparticle formulation.[56] In the same group, PLGA nanoparticles were used intravenously to deliver pitavastatin in mice.[57] Injected nanoparticles were rapidly taken up predominantly by monocytes and macrophages. This study showed that nanoparticles accumulated in macrophages in atherosclerotic plaques; this might be an important consideration if nanoparticles are to be used in cases of advanced atherosclerosis. Assays using cultured macrophages showed that delivery using nanoparticles resulted in enhanced cellular uptake and retention over 1 week.

Apart from the examples we have mentioned in which siRNA has been delivered using liposomal formulations, it will be interesting to see whether targeted delivery is beneficial for other anti-inflammatory treatments; for example, nanoparticle-based delivery might assist in targeting one particular population of macrophages among those present in the heart. Because inflammation is important in clearing cellular debris in preparation for tissue repair, indiscriminate suppression of the inflammatory response will most likely be detrimental, but a 'measured approach' could offer improvements in reducing acute ischemia-reperfusion damage.[50]

Other mechanisms

A diverse collection of pharmacological agents, including adenosine, erythropoietin, morphine, numerous peptides and growth factors, nicorandil, and transforming growth factor- β , have shown cardioprotective effects in experimental models of cardiac ischemia–reperfusion.[36] Of these agents, some suffer from very short half-life *in vivo* owing to degradation, metabolism, or enzymatic degradation.[58] The mechanisms of action include conferring protection against apoptosis, increasing oxygen supply or decreasing demand, and modulating signalling and ion transport. While growth factors have been investigated for their ability to initiate angiogenesis and repair of the myocardium, some have also been shown to be cardioprotective in models of ischemia–reperfusion.[59]

Adenosine is released during ischemia and adenosine receptor agonists are cardioprotective when given during ischemia or reperfusion. Work in Jacobson's group shows that the branched structure of a PAMAM dendrimer can be functionalized with adenosine receptor agonists to form a multivalent and thus highly potent cardioprotective therapeutic agent. The effects of three different receptor agonist-modified dendrimers in hypoxic cardiomyocytes, cardiomyocytes treated with H_2O_2 , and *ex vivo* hearts subjected to ischemia–reperfusion were investigated.[60, 61] Adenosine has also been encapsulated in liposomes (130 nm).[62] Compared to free adenosine, administration of the liposomal formulation prior to reperfusion in rats prolonged time in circulation and delayed degradation, showed greater accumulation in infarcted myocardium, and decreased effects on hemodynamic parameters. Wan *et al.* investigated the use of PAMAM G4 dendrimer conjugates (~4.5 nm) to deliver adenosine receptor agonists in *ex vivo* Langendorff-perfused murine hearts. The conjugates in this work were designed to remain intact rather than release free drug; the authors confirmed that potency of the drug was retained by the conjugate. In fact, because the dendrimers were functionalized with multiple drug moieties, multivalent interactions with these G protein-coupled receptors actually improved potency of the agonists. In contrast to most other studies, conjugates were infused prior to the induction of no-flow ischemia so the effectiveness of the drug in an acute clinical setting is difficult to predict. As might be expected, the extent of extravasation from the microvasculature decreases with increasing size, most noticeably between third and fourth generation dendrimers.[63] Galagudza *et al.* made use of the ability of nanoparticle formulations to protect rapidly metabolized adenosine and achieve delivery in a targeted way. The authors suggest its use for ischemic post-conditioning, but this is problematic because adenosine has a half-life of less than 10 s in blood, and can induce hypotension at required doses that may preclude its clinical use. When adenosine was immobilized on the surface of silica nanoparticles, the hypotensive effect was reduced and a

decrease in infarct size was reported compared to the free drug.[12] Chang *et al.* investigated PLGA particles of size 60 nm, 200 nm, and 1 μm as a vehicle for the delivery of insulin-like growth factor 1 (IGF-1). Unlike other studies, in this work, the therapeutic growth factor was electrostatically bound to the particle surface, instead of being encapsulated. For this reason, and due to the increasing surface area-to-volume ratio with decreasing particle size, the greatest loading of IGF-1 was achieved on the smallest of these particles. Labelling of PLGA nanoparticles with quantum dots revealed a retention time of 1–3 days following peri-infarct injection in a murine permanent LAD ligation model. Nanoparticle administration reduced doxorubicin-induced cardiomyocyte apoptosis compared to free IGF-1, and improved functional outcomes at 21 days post-injury.[64] Binsalamah *et al.* injected chitosan-alginate nanoparticles directly into the myocardium to deliver placental growth factor (PlGF). Nanoparticle protected the growth factor against degradation and enabled sustained release to stimulate angiogenesis in a rat ischemia–reperfusion injury.[58]

Serpooshan *et al.* used PEGylated liposomes to protect apelin peptides against degradation (the plasma half-life is <8 min). Apelin is a peptide ligand for a G-protein-coupled receptor that ameliorates cardiac dysfunction and remodeling. Compared to free peptide, the liposomal formulation displayed enhanced efficacy and sustained release of drug *in vitro* and *in vivo*, and reduced left ventricular hypertrophy and pressure overload-induced cardiac dysfunction in a mouse model of transverse aortic constriction.[65]

Nucleic acids, including plasmids and siRNA, can be used to up- or down-regulate production of various growth factors and proteins, but delivery requires some means of protection against degradation by enzymes while facilitating cellular internalization.[66] In some cases, the suppression of pathology at the gene level can be more effective than pharmacological inhibition, because feedback may upregulate expression in response to drug treatment.[67] VEGF plasmids can be delivered in nanoparticle form using non-viral transfectants such as PAMAM dendrimers,[68, 69] and transfection of myoblasts in the local cellular environment can be achieved using polycaprolactone/polyethylenimine electrospun nanofibers.[70] A dendron-based delivery system for siRNA consisting of G4 PAMAM attached to PEG and a CPP (R_9 or TAT) to target cardiomyocytes has been reported by Liu *et al.*[67]. The LAD artery was occluded for a period of 30 min before reperfusion in adult Sprague-Dawley rats and siRNA was used to silence expression of the angiotensin II type 1 receptor (AT1R). Echocardiography and hemodynamic measurements revealed improvements in ejection fraction, end systolic volume, and end diastolic volume after 3 days.

Limitations and opportunities in treating the ischemic myocardium

There are many problems associated with treatment of ischemic/reperfusion injury. First, biochemical changes induced by ischemia–reperfusion begin within minutes of reperfusion,[5] which makes for a very short window of opportunity; second, drug therapy is limited by access to the ischemic myocardium, because blood flow in ischemia is some 10^6 times lower than normal[71] and owing to ‘washing out’ of therapies by contraction of myocardium;[72] and third, drugs and particles introduced into the circulation generally do not have an innate propensity to accumulate in the myocardium. Nevertheless, in accordance with the mechanisms of damage discussed up to this point, delivery of therapies that modulate ROS production or scavenging, Ca^{2+} flux, and inflammation in cardiomyocytes, the myocardial vasculature, or mitochondria have all been identified as potential cardioprotective interventions. It is important to note that targeting of nanoparticles to mitochondria (and indeed, to other subcellular organelles) can be disrupted by the endosomal pathway, because endosomes are a barrier to mitochondrial trafficking, and that not all types of nanoparticles can cross the mitochondrial double membrane.[73] There are a variety of drugs that might aid in treating ischemia, but many such compounds are limited by a short half-life and poor solubility, or lack of specificity and retention in the myocardium with a tendency to induce severe systemic effects.[13, 71] These treatments are ideal candidates for targeted drug delivery. A targeted delivery mechanism could assist in maximizing therapeutic effect while limiting side effects in healthy tissue,[4] and assist in overcoming the difficulties in treating the ischemic myocardium.

Nanoparticles, including lipid-based (micelles and liposomes), polymer-based systems (dendrimers, polyplexes, nanospheres) as well as other novel nano-sized architectures, constitute an attractive route for improving the delivery of therapies to the myocardium for several reasons. They can decrease toxicity and improve solubility and stability of drugs,[4] they can remain in the circulation without the risk of blocking blood vessels,[6, 64, 74] they can target therapies to specific cell types[4, 13] or even to specific organelles,[75] and they can prolong therapeutic effects by modulating the release of drug.[6, 76] One of the main advantages is that nanoparticle systems can deliver multiple therapies and also facilitate imaging in a targeted fashion. Additionally, ‘smart’ nanomaterials can be designed to respond to external stimuli, releasing drugs on demand, or in response to pathology.[77] Interestingly, extracellular vesicles secreted by human cardiac progenitor cells are themselves nanoparticles, containing proteins, mRNAs and miRNAs encapsulated in a lipid bilayer membrane that inhibit apoptosis, promote angiogenesis, and exhibit other cardioprotective effects in myocardial injury.[78-81]

Fortunately, current treatment of acute myocardial ischemia with percutaneous coronary intervention provides an opportunity for administration of nanoparticle-based therapies. At the time of reperfusion, distribution of nanoparticles is extended due to increased blood flow. The early stages of reperfusion contribute strongly to injury,[12] and microvascular permeability can induce an inflammatory response at this time, so reperfusion is a critical time at which therapies can be practically delivered to the myocardium (Figure 2).

In light of the tendency of the heart to circulate injected therapeutics throughout the body, there is a need for immobilization/targeting strategies in the myocardium, particularly in the acute phase of treatment. Given the short window of opportunity in treating some of the effects of acute ischemia, direct introduction of nanomaterials into the myocardium during percutaneous coronary intervention may be a more useful strategy than intravenous delivery, which could delay delivery through accumulation time or slow release of nanoparticle cargo.[19] It is important to note that cardiomyocytes are non-phagocytic cells so do not immediately lend themselves to particle uptake.[64] While microparticles can also be used for immobilization of therapies, particle size is an important consideration, affecting tissue penetration and the rate of cellular internalization.[82]

In the longer term, intravenously delivered nanoparticle-based delivery systems can improve drug targeting via mechanisms that are selectively involved in ischemia–reperfusion injury but not in healthy tissue.[6] Targeting therapies to pathological sites can reduce systemic toxicity and improve efficacy, particularly in cases where drug doses are limited by systemic toxicity or effects on peripheral vasculature.[4] During ischemia, the cardiovascular endothelium is subject to hypoxia and becomes vulnerable, leaky, and eventually necrotic.[3] In accordance with this change, the accumulation of particles can be facilitated in ischemic tissue as capillary extravasation increases (passive targeting).[6] Active targeting strategies, where particles are modified with ligands with an affinity to a uniquely expressed biomarker of injury, can also be used to selectively direct therapies.[13] Relying on accumulation of particles from the circulation generally takes 24–72 h, which could be too slow for treatment in acute ischemia. Nevertheless, some studies show that cardioprotective agents delivered up to 24 hours into myocardial reperfusion may still limit acute ischemia–reperfusion damage 72 hours post-injury.[5]

In summary, cardiac ischemia–reperfusion injury is a highly complex process that would benefit from a range of simultaneous therapeutic approaches. Nanoparticles can be used to deliver multiple therapies in a highly specific way, and it is this benefit that may lead to novel, innovative treatments not only for ischemia–reperfusion injury, but cardiovascular diseases in general. Future perspective

From our discussion, it is clear that there has been significant progress in improving delivery of drugs to the myocardium using nanotechnology, of which we have provided some recent examples. Of course, there are still considerable challenges to overcome in realizing nanoparticle-mediated delivery for the treatment of ischemia–reperfusion injury. Size and charge are just two factors that affect the myocardial retention of nanoparticles, and targeting and immobilization strategies are likely to improve the prospects of nanoparticle-based treatments in the short term. The type of nanoparticle is also important in this regard. Additionally, nanoparticles must have low immunogenicity, avoid causing further complications such as arrhythmia[41], not exacerbate inflammation, and must be either excreted or degraded once they have fulfilled their task.[83] Research in these areas is still relatively unexplored in the myocardial context. Nevertheless, despite these challenges, studies showing potential improvements in targeting and bioavailability suggest that re-examining previously ineffective pharmacological treatments is worthy of investigation.

While intramyocardial injection is the preferred method in small experimental animals, this is unlikely to be the final goal in translation to the clinic, owing to the instability of damaged tissue following myocardial infarction.[84] Intravenous or intracoronary injection would be ideal, so further improvements in targeting (particularly speed and specificity) are critical for treating the reperfused myocardium. While a range of peptides can be used to target specific organelles[85] and cells[19], and selective markers of injury can facilitate active targeting[13], mechanisms for preventing uptake by the liver and spleen still require further work. Magnetic nanoparticles may provide a useful means of targeting not only for retention of stem cells in the myocardium, but also for particle delivery.[86-88] Nanoparticles bearing cationic dendrons via biodegradable linkers might also be useful for improving immobilization within the myocardium in the acute phase followed by detachment and vascular targeting after some time. Mitochondrial targeting of nanoparticles remains a difficult aim, although a number of particles have been successfully directed to mitochondria.[89] The majority of mitochondrial-targeting particles make use of the triphenylphosphine ligand, which facilitates targeting by its hydrophobicity and the high membrane potential, but peptide-based targeting is also a possibility.[90] Mitochondrial nano-

sized delivery constructs have been reviewed elsewhere and the targeting of mitochondria in acute ischemia remains an area of ongoing research.[34, 91]

It is also apparent that cardiac ischemia–reperfusion is a multifaceted problem, and would likely benefit from a combination of the approaches we have discussed here, in addition to imaging and diagnosis. Although such combinations are common in cancer therapy, their use in cardiovascular therapy is less widespread. An obvious question that follows is why smart or multifunctional nanoparticles, which offer some of the most significant advantages of nanotechnology, have not been extensively exploited in cardiac research. In particular, the ability to create nanoparticles that can deliver multiple cargoes and facilitate multiple imaging modalities remains an unexplored area. In addition to acute ischemia–reperfusion injury, ideal therapies for MI need to also produce new cardiomyocytes and initiate revascularization to the infarcted region.[58] Using acute and chronic therapeutic strategies simultaneously (e.g., protecting vulnerable cardiomyocytes while initiating repair), or making use of oxygen-bearing perfluorinated components while simultaneously delivering calcium channel inhibitors, will undoubtedly lead to some innovative findings.

In the long run, transfection may be a useful approach to combine with treatments for acute injury. Transfection would enable gene therapy for repair of the damaged myocardium. The use of viral vectors is not ideal for this purpose. Additionally, delivery of a plasmid might be useful for short-lived or unstable growth factors in treating injury. On a higher level, transfection of cardiomyocytes using magnetic nanoparticles functionalized with non-viral vectors could result in controllable transfection to treat a range of cardiovascular disorders, many of which are related to single gene mutations.[92]

In attempting to prevent the progression from cardiac ischemia to hypertrophy and heart failure, ameliorating the effects of acute ischemia–reperfusion injury should be regarded as a priority before regenerative treatments are considered. Many of the key players in ischemia–reperfusion injury have been reasonably well characterized, and these suggest a number of putative therapeutic targets. In this respect, combining therapies that focus on reducing ROS-induced damage and subsequent Ca^{2+} mishandling with the benefits of nanotechnology will likely result in some improvements where these therapies have failed in the past.

Additionally, the ability to target mitochondria and direct drugs and biomolecules to specific cell types will likely open new therapeutic avenues for treating acute ischemia–reperfusion injury. We suggest that smart nanomaterials and multifunctional nanoparticles combining imaging, targeting, gene therapy and the controlled release of drugs may be a particularly useful approach in addressing acute ischemia–reperfusion, which remains an area in which there is potential for therapeutic improvement in cardiovascular disease.

Reference annotations

[Ref 4] * Broad introductory review of types of nanoparticles and their uses in delivering therapies to the myocardium

[Ref 5] ** Review of acute myocardial ischemia–reperfusion injury, including pathophysiology, mediators of injury, and therapeutic strategies

[Ref 11] ** Review of the use of micro- and nanoparticles in treating cardiovascular disease (including myocardial infarction and peripheral artery disease)

[Ref 21] * Review describing delivery to mitochondria using nanoparticles

[Ref 34] * Review describing delivery to mitochondria using nanoparticles

[Ref 14] ** Comparison of the accumulation of micelles and liposomes in the myocardium using passive targeting

[Ref 40] * Example of simultaneous delivery of calcium channel antagonist and antioxidant using polymeric nanoparticles

[Ref 51] * Example of simultaneous delivery of anti-inflammatory drugs and plasmids

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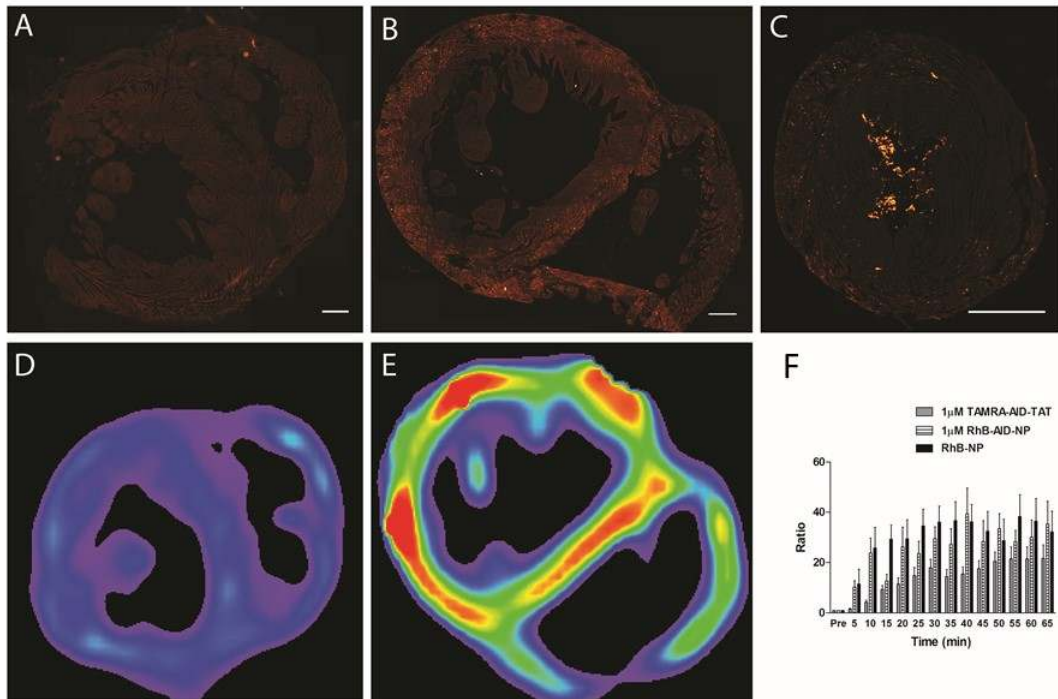
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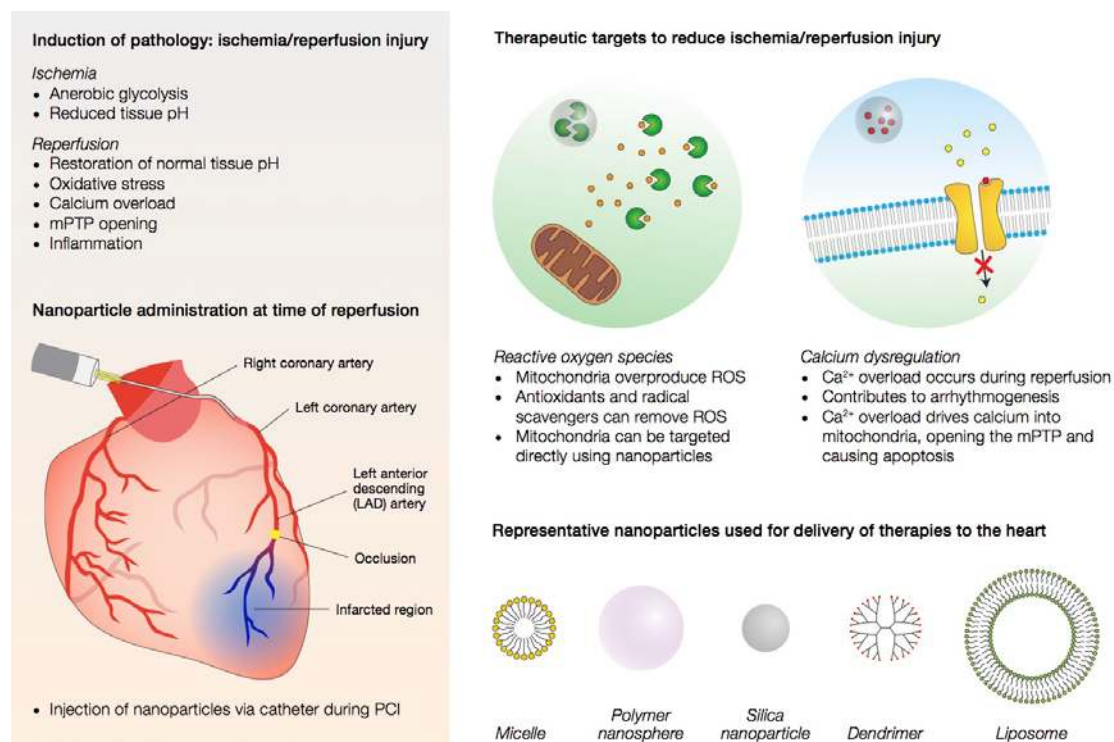
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Figure 1



Assessment of bio distribution of AID in hearts perfused with either AID-nanoparticles (AID-NP) or AID-TAT labelled with the fluorescent indicator TAMRA (TAMRA-AID-TAT). (A) Fluorescent microscopy of a heart perfused with Krebs buffer only for 30 min. (B) Fluorescent microscopy of a heart perfused with Krebs buffer supplemented with 1 μM AID-NP for 30 min. (C) Fluorescent microscopy of a heart perfused with Krebs buffer supplemented with 1 μM AID-TAT-TAMRA for 30 min. (D) R2 MRI (magnetic resonance imaging) map of heart perfused only with KHB (i.e., heart shown in panel A) for 30 min. (E) R2 MRI map of heart perfused with Krebs buffer supplemented with 1 μM AID-NP (i.e., heart shown in panel B) for 30 min. Note the brighter, higher R2 values, indicating accumulation of nanoparticles. (F) Fluorescent uptake of rhodamine labelled AID-tethered nanoparticles (RhB-AID-NP) and TAMRA-AID-TAT into cardiac myocytes over time. Minimum n = 12 per time point, per sample. (TAMRA and Rhodamine have identical fluorescence excitation and emission spectra). Adapted from ref [38].

Figure 2



A summary of key pathological changes during acute cardiac ischemia, proposed therapeutic targets, and nanoparticle architectures for treatment of ischemia–reperfusion injury. Following myocardial infarction (necrosed area distal to block of coronary artery), injection into the coronary artery via catheter at the time of percutaneous coronary intervention (PCI) is the preferred method of nanoparticle administration to treat ischemia–reperfusion injury. Nanoparticle-based delivery can offer improvements in delivery of therapeutics to address increased reactive oxygen species production and calcium dysregulation, in addition to inflammation and other mechanisms (see text for further information). A range of nanoparticle architectures, including micelles and liposomes, polymer nanoparticles, inorganic nanoparticles (including silica and quantum dots), and dendrimeric structures, are shown.

Table 1. Summary of literature showing therapies delivered to the heart using nanoparticles.

Drug/payload	Drug type	Nanoparticle (composition, targeting)	Model	Reference(s)
Sodium selenite	Antioxidant	Polymer NPs (guar gum)	H9c2 rat cardiomyoblasts, 30 min O ₂ -free incubation	[27]
Phenyl-2-aminoethyl selenide	Antioxidant	Liposomes	Nude mice, PC3 xenograft, protection against cardiac damage induced by doxorubicin	[28]
Resveratrol + quercetin	Antioxidant	Micelles (Pluronic F-127)	Protection of H9c2 cardiomyocytes against doxorubicin	[30]
Coenzyme Q10 (ubiquinone)	Antioxidant	Polymer NPs (PLGA)	LAD coronary artery ligation in Sprague-Dawley rats; oral administration	[32]
Nitric oxide	ROS	Dendrimer (G4 PAMAM)	<i>Ex vivo</i> rat hearts; no-flow ischemia + reperfusion	[31]
AID peptide	L-type calcium channel antagonist	Polymer NPs (PGMA)	<i>Ex vivo</i> guinea pig hearts; no-flow ischemia + reperfusion	[38]
AID peptide + curcumin, resveratrol	L-type calcium channel antagonist, antioxidants	Polymer NPs (PGMA)	<i>Ex vivo</i> rat hearts, mouse ventricular myocytes	[40]
Cyclosporine A	mPTP inhibitor	Polymer NPs (PLGA)	Cultured rat cardiomyocytes, murine <i>in vivo</i> model	[37]
S100A1	Calcium-binding protein	Polymer NPs (polyketal, targeted with N-acetylglucosamine)	Rat ventricular myocytes, pulmonary artery banding-induced heart failure	[44]
Rapamycin	Immunosuppressant	Micelles (targeted with cRGD)	<i>In vitro</i> endothelial cells, <i>ex vivo</i> mouse aorta	[55]
Pitavastatin	Statin	Polymer NPs (PLGA)	Rat transient occlusion of LAD coronary artery, murine model of atherosclerosis	[56, 57]
Insulin-like growth factor-1	Growth factor	Polymer NPs (PLGA)	Peri-infarct injection in murine LAD ligation	[64]
Placental growth factor	Growth factor	Polymer NPs (chitosan-alginate)	Myocardial injection following LAD ligation in rat	[58]
[Pyr1]-apelin-13	Polypeptide	Liposomes	Murine model of hypertrophy (transverse aortic constriction)	[65]
(Nox2)	siRNA	Polymer NPs (polyketal)	Macrophages <i>in vitro</i> , murine LAD artery ligation	[25]
(CCR2)	siRNA	Liposomes	Murine transient LAD occlusion	[54]
(Interferon regulatory factor 5)	siRNA	Lipid-based NPs	Murine LAD ligation	[53]
(angiotensin II type 1 receptor)	siRNA	Dendrimer (G4 PAMAM, targeted with R ₉ or TAT)	LAD transient occlusion	[67]
VEGF	Plasmid	Dendrimeric bio-reducible polymer (based on PAMAM)	Rat transient LAD artery occlusion	[68]
VEGF	Plasmid	Dendrimeric/branched polymer (PEI)	Rabbit first branch of left circumflex coronary artery ligation	[69]
VEGF	Plasmid	Nanofibers (PCL/PEI)	H9c2 myoblasts	[70]