

2004

## Controlled Release Systems for DNA Delivery

Angela K. Pannier

*University of Nebraska-Lincoln*, [apannier2@unl.edu](mailto:apannier2@unl.edu)

Lonnie D Shea

*Northwestern University*, [l-shea@northwestern.edu](mailto:l-shea@northwestern.edu)

Follow this and additional works at: <https://digitalcommons.unl.edu/biosysengfacpub>



Part of the [Biological Engineering Commons](#)

---

Pannier, Angela K. and Shea, Lonnie D, "Controlled Release Systems for DNA Delivery" (2004). *Biological Systems Engineering: Papers and Publications*. 158.

<https://digitalcommons.unl.edu/biosysengfacpub/158>

This Article is brought to you for free and open access by the Biological Systems Engineering at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Biological Systems Engineering: Papers and Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

REVIEW

# Controlled Release Systems for DNA Delivery

Angela K. Pannier<sup>1</sup> and Lonnie D. Shea<sup>2,3,4</sup>

1. Department of Interdepartmental Biological Sciences, Northwestern University

2. Department of Chemical and Biological Engineering, Northwestern University

3. Department of Biomedical Engineering, Northwestern University,

4. Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, IL 60611, USA

*Corresponding author* – Lonnie D. Shea, 2145 Sheridan Road, E156, Evanston, IL 60208-3120, USA;  
fax 847 491-3728, e-mail [l-shea@northwestern.edu](mailto:l-shea@northwestern.edu)

## Abstract

Adapting controlled release technologies to the delivery of DNA has the potential to overcome extracellular barriers that limit gene therapy. Controlled release systems can enhance gene delivery and increase the extent and duration of transgene expression relative to more traditional delivery methods (e.g., injection). These systems typically deliver vectors locally, which can avoid distribution to distant tissues, decrease toxicity to nontarget cells, and reduce the immune response to the vector. Delivery vehicles for controlled release are fabricated from natural and synthetic polymers, which function either by releasing the vector into the local tissue environment or by maintaining the vector at the polymer surface. Vector release or binding is regulated by the effective affinity of the vector for the polymer, which depends upon the strength of molecular interactions. These interactions occur through nonspecific binding based on vector and polymer composition or through the incorporation of complementary binding sites (e.g., biotin-avidin). This review examines the delivery of nonviral and viral vectors from natural and synthetic polymers and presents opportunities for continuing developments to increase their applicability.

**Keywords:** gene therapy, tissue engineering, biomaterials, substrate-mediated delivery, plasmid

## Introduction

Controlled release systems for low-molecular-weight drugs and proteins have become a multibillion dollar industry, with products such as Nutropin Depot, Gliadel wafer, Norplant, and CYPHER Stent. These systems illustrate the potential advantages of controlled release, which include: (i) drug levels maintained within a desirable range, (ii) localized delivery to a target tissue or cell type to avoid adverse side effects, (iii) decreased dose or number of dosages, and (iv) facilitated delivery for fragile compounds (i.e., short half-lives). The adaptation of controlled release technologies to the delivery of nonviral and viral vectors has the potential to overcome extracellular barriers that limit gene therapy. Controlled release can maintain elevated DNA concentrations in the cellular microenvironment, which improves gene delivery.<sup>1</sup> Localized vector delivery to specific tissues can avoid distribution to distant tissues, leading to ectopic gene expression, toxicity to nontarget cells, and a strong immune response. Additionally, viral and nonviral vectors may have a relatively short half-life,<sup>2–4</sup> and delivery vehicles can prevent their degradation and/or provide

a sustained release. This review examines the mechanisms of gene delivery from biomaterials and discusses how continuing advances will increase the applicability of controlled release to gene delivery.

## Delivery Mechanisms

Controlled release systems typically employ polymeric biomaterials that deliver vectors according to two general mechanisms: (i) polymeric release, in which the DNA is released from the polymer, or (ii) substrate-mediated delivery, in which DNA is retained at the surface. For polymeric release, DNA is entrapped within the material and released into the environment, with release typically occurring through a combination of diffusion and polymer degradation. Polymeric delivery may enhance gene transfer by first protecting DNA from degradation and then maintaining the vector at effective concentrations, extending the opportunity for internalization. DNA release into the tissue can occur rapidly, as in bolus delivery, or extend over days to months.<sup>5–7</sup> For rapid release, levels would be expected to rise quickly and decline as the DNA is cleared or degraded. For sus-

tained delivery, the concentration may be maintained within an appropriate range by adjusting the release to replace DNA that is cleared or degraded. Conversely, substrate-mediated delivery, also termed solid-phase delivery, describes the immobilization of DNA to a biomaterial or extracellular matrix, which functions to support cell adhesion as well as migration and places DNA directly in the cellular microenvironment. The immobilization of DNA to the matrix may seem counterintuitive given the need for cellular internalization to achieve expression; however, natural and synthetic corollaries exist for growth factors and viral vectors. Growth factors associate with the extracellular matrix, functioning directly from the matrix or upon release.<sup>8</sup> Additionally, many viral vectors associate with the extracellular matrix as a means to facilitate cellular binding and internalization.<sup>9, 10</sup> In substrate-mediated delivery, DNA is concentrated at the delivery site and targeted to the cells that are adhered to the substrate.<sup>11, 12</sup> Cells cultured on the substrate can internalize the DNA either directly from the surface or by degrading the linkage between the vector and the material.

Molecular interactions between the vector and the polymer dictate whether the DNA is released from or bound to the delivery vehicle. Viral and nonviral vectors, which contain negatively charged DNA or RNA potentially complexed with proteins, cationic polymers, or cationic lipids, interact with polymeric biomaterials through nonspecific mechanisms, including hydrophobic, electrostatic, and van der Waals interactions. These interactions have been well characterized for adsorption and release of proteins from polymeric systems,<sup>13, 14</sup> Nonspecific binding depends upon the molecular composition of the vector (e.g., lipid, polymer, protein) and the relative quantity of each (e.g., N/P). Alternatively, specific interactions can be introduced through complementary functional groups on the vector and polymer, such as antigen-antibody or biotin-avidin, to control vector binding to the substrate. The effective affinity of vector for polymer is determined by the strength of these molecular interactions, which may also be influenced by environmental conditions (e.g., ionic strength, pH), binding-induced conformational changes, or vector unpacking. Delivery from most polymeric systems likely occurs through a combination of binding and release mechanisms, and both the vector and the polymer can be designed to regulate these interactions.

### Vehicle Formulations

Vehicles for gene delivery can be fabricated from both natural and synthetic polymers and processed into a variety of forms, including nanospheres, microspheres, or scaffolds. Nanospheres are particles with diameters ranging from approximately 50 to 700 nm,<sup>15</sup> consistent with the size of viral and nonviral vectors. Nanoparti-

cles are internalized and release DNA intracellularly. In contrast, microspheres, with diameters ranging from 2 to 100  $\mu\text{m}$ , are not readily internalized, but retained within the tissue to release DNA.<sup>16, 17</sup> Released DNA can transfect cells at the delivery site, with the protein product acting locally or distributed systemically.<sup>17, 18</sup> Alternatively, polymeric scaffolds function to define a three-dimensional space and can either be implanted or be designed to solidify upon injection. These scaffolds can deliver DNA to cells within the surrounding tissue or can target those cells infiltrating the scaffold.<sup>7, 18</sup> The scaffold can also distribute the vector throughout a three-dimensional space, and transfection on a three-dimensional construct may extend transgene expression.<sup>19</sup>

A variety of natural and synthetic materials have been employed for DNA delivery, which can be categorized as either hydrophobic [e.g., poly(lactide-co-glycolide) (PLG), polyanhydrides] or hydrophilic polymers [e.g., hyaluronic acid (HA), collagen, poly(ethylene glycol) (PEG)]. Synthetic polymers such as PLG and polyanhydrides have been widely used in drug delivery applications, as they are biocompatible and available in a range of copolymer ratios to control their degradation.<sup>20</sup> Drug release from these polymers typically occurs through a combination of surface desorption, drug diffusion, and polymer degradation.<sup>21</sup> For DNA delivery, polymer processing techniques are being developed to fabricate a range of geometries and properties while retaining the activity of the vector during processing and release.<sup>22, 23</sup> Alternatively, mild processing conditions can be employed to process hydrophilic polymers into hydrogels. These hydrogels are often more than 98% water and maintain the activity of encapsulated vectors, which are released by diffusion from the polymer network.<sup>6</sup> Crosslinking the polymer or increasing the density can slow the release and allow network degradation to control the rate.<sup>24-26</sup> These hydrophilic polymers, along with some hydrophobic polymers, contain functional groups (e.g., carboxylic acids, amines) in the polymer backbone that can be readily modified, allowing interactions between the polymer and the vector to be manipulated.

### Plasmid delivery

Plasmid DNA delivery by physical methods generally results in low but sustained expression *in vivo*, which is limited by poor uptake due to factors such as degradation and clearance.<sup>27, 28</sup> Physical (e.g., ultrasound, hydrodynamic injection) methods are continually being improved to enhance cellular uptake of DNA by altering cell permeability.<sup>27, 29</sup> Plasmid uptake may involve intrinsic cellular processes, but the processes governing intracellular transport remain elusive. Following delivery to the nucleus, expression can typically occur over

time scales of days to weeks or months.<sup>29</sup> Extracellular factors that limit delivery include plasmid clearance or degradation, which can be mediated by sequence-specific recognition from the immune system. Immune responses to the plasmid are affected by the methylation pattern of CpG sequences that can affect the duration of transgene expression.<sup>29</sup> Polymeric delivery represents an alternative approach that can increase residence time within the tissue and protect against degradation. Plasmids ( $10^3$ – $10^4$  bp) have effective hydrodynamic diameters in excess of 100 nm and a highly negative surface charge density.<sup>30</sup> The large size of the DNA limits transport through tissues, resulting in diffusion coefficients on the order of  $10^{-9}$  to  $10^{-12}$  cm<sup>2</sup>/s,<sup>31</sup> and promotes localized delivery when polymers are inserted into a tissue.<sup>10</sup> In the following paragraphs, we limit our discussion of polymeric delivery systems primarily to plasmid delivery, though these systems may also benefit oligonucleotide or siRNA delivery.<sup>32</sup>

Plasmid DNA interacts weakly with many polymers, leading to *in vitro* release from the vehicle with rates modulated by the polymer properties. Many synthetic and natural polymers are negatively charged, and thus the weak interactions likely result from repulsive charge interactions between plasmid and polymer. Collagen-based materials release plasmid DNA for times ranging from hours to days.<sup>6, 24</sup> HA-based hydrogels also release the DNA; however, the rate of release can be controlled by the extent of crosslinking.<sup>26, 33</sup> Sustained release and delayed release was achieved with hydrogels composed of PEG with lactic acid segments, based on the number of degradable lactic acid units.<sup>25</sup> For hydrogels formed by crosslinking, the approach must be adjusted to avoid damaging the integrity of the DNA.<sup>25</sup> For synthetic polymers such as PLG, the integrity of the DNA can be affected by degradation of the polymer to lactic acid and glycolic acid.<sup>7</sup> PLG polymers can provide release rates ranging from a few days to more than 60 days, with the fabrication method and the polymer composition regulating release.<sup>7, 23, 34, 35</sup> Ethylene vinyl-co-acetate (EVAc) polymers can similarly provide a sustained release of DNA on the time scale of weeks.<sup>36, 37</sup>

DNA-releasing polymers administered to multiple sites *in vivo* have demonstrated the capacity to transfect cells locally and promote sustained protein production.<sup>18</sup> Although successful gene transfer has been achieved, the design parameters that relate system properties to the quantity and duration of transgene expression are not well understood. For example, an injectable PLG formulation delivered subcutaneously led to 28 days of expression with 50  $\mu$ g of DNA.<sup>34</sup> An implantable PLG scaffold delivering 500  $\mu$ g of DNA was able to transfect cells within and adjacent to the scaffold.<sup>7</sup> EVAc disks implanted intravaginally induced gene expression for 28 days with 45  $\mu$ g of DNA.<sup>36</sup> Collagen minipellets containing 50  $\mu$ g of DNA administered intramuscularly elicited

systemic effects for at least 60 days, which was significantly longer than direct DNA injection.<sup>17</sup> Similarly, collagen implanted into a bone defect transfected cells locally with 1 mg of DNA. Controlled studies to correlate scaffold design with *in vivo* gene delivery are needed to optimize the development of delivery systems.

Nanospheres loaded with plasmid DNA represent an alternative to the traditional complexation agents. PLG nanoparticles are internalized by cells and a fraction escape the endosome within 10 min of incubation, which is proposed to occur through charge reversal.<sup>38</sup> The DNA likely diffuses from the pores of the nanoparticles, as opposed to decomplexing from a cationic polymer or lipid. Transfection levels achieved *in vitro* with these nanoparticles are significantly lower than with a bolus delivery of lipoplexes.<sup>39, 40</sup> However, a substantial increase in expression was observed through 1 week of culture, suggesting that the nanospheres provided a controlled release intracellularly. Nanospheres fabricated from natural polymers (gelatin, chitosan) provided transfection that was comparable to that obtained by Lipofectamine.<sup>41</sup> Gelatin was crosslinked to stabilize the nanospheres and provided some protection against nuclease degradation.<sup>42</sup> Intramuscular delivery of these nanospheres produced higher and more sustained expression than plasmid DNA.

Although DNA is typically encapsulated and released from materials, cationic groups can be attached to the material to promote DNA binding. Collagen was cationized through modification with amino groups or polylysine<sup>43</sup> and degradation of the collagen led to release of the bound DNA. Alternatively, PEI (polyethylenimine) or polylysine was bound to or blended with the material,<sup>44, 45</sup> resulting in DNA binding and some cellular transfection *in vitro*. Similarly, plasmid DNA adsorbed onto PLG microparticles coated with the cationic surfactant cetyltrimethylammonium bromide was able to transfect dendritic cells *in vitro*.<sup>46</sup> Binding of plasmid DNA to a cationic material may exhibit limited cellular internalization due to the strong interactions between the DNA and the material. The alternative is to allow the DNA to form complexes in solution and then immobilize the complexes to the polymers.

## DNA Complex Delivery

Although plasmid DNA provides transfection *in vivo*, packaging DNA with cationic lipids or polymers can facilitate uptake and transfection *in vitro* and *in vivo*.<sup>27, 29, 47</sup> Cationic polymers and lipids protect DNA against degradation by nucleases and serum components, create a less negative surface charge, and can be designed to target delivery to specific cell types through receptor–ligand interactions.<sup>47</sup> These complexation agents can also facilitate intracellular trafficking, which includes endo-

somal escape, cytoplasmic transport, and nuclear entry, while also dissociating from the DNA to allow expression.<sup>27, 48</sup>

Polymeric release of DNA complexes may enhance or localize gene transfer *in vivo* and *in vitro*; however, interactions between the vector and the polymer can impact incorporation and release. The presence of complexation agents can maintain the stability of DNA complexes during polymer processing<sup>49, 50</sup> and in some cases increase encapsulation efficiency.<sup>32</sup> Porous PLG or collagen scaffolds with encapsulated polyplexes or lipoplexes achieved substantial transfection *in vitro*<sup>6, 45, 51</sup> and *in vivo*,<sup>6</sup> but with significantly altered release profiles compared to plasmid DNA. The complexation reagents interact with the biomaterial or with adsorbed serum components, which are known to interact with DNA complexes.<sup>52, 53</sup> PEI/DNA complexes and lipoplexes were slowly released from collagen, and addition of a protective copolymer to the complexes increased release, presumably by decreasing interactions between collagen and the complex.<sup>6</sup> While low N/P ratios supported release from synthetic polymers,<sup>50</sup> high N/P ratios significantly limited release.<sup>32, 51</sup> Interestingly, these vector-polymer interactions resulted in released complexes with a lower N/P ratio than the encapsulated complexes.<sup>54</sup>

Interactions between complexation agents and the polymer have been adapted to immobilize DNA complexes specifically to a substrate. Poly(L-lysine) (PLL) and PEI were modified with biotin residues for subsequent complexation with DNA and binding to a neutravidin substrate.<sup>12, 55</sup> Complexes were formed with mixtures of biotinylated and nonbiotinylated cationic polymer at a constant N/P ratio. Release studies demonstrated that only 25% of immobilized DNA complexes were released over an 8-day period, with approximately 15% released within the first 24 h. For complexes formed with PLL, the number of biotin groups and their distribution among the cationic polymers were critical determinants of both binding and transfection. The number of biotin groups in the complex was manipulated through the fraction of biotinylated PLL used for complex formation and the number of biotin residues per PLL. Increasing the number of biotin groups per complex led to increased binding.<sup>12</sup> However, *in vitro* transfection was maximal when complexes contained biotin residues attached to a small fraction of the cationic polymers.<sup>55</sup> At this condition, less than 100 ng of immobilized DNA mediated transfection, which was increased 100-fold relative to bolus delivery of similar complexes.<sup>12</sup> Additionally, transfection was observed only in the location to which complexes were bound, suggesting the possibility of spatially regulating DNA delivery. For complexes formed with PEI, substantial transfection was observed *in vitro* but was independent of the number of biotin groups present on the complex, suggesting that complex immobilization occurred through nonspecific interactions.<sup>55</sup>

Nonspecific binding of DNA complexes to substrates has been employed with other systems to mediate delivery. PLGA and collagen membranes were coated with phosphatidyl glycerol (1–5%) to support binding of complexes formed with polyamidoamine dendrimers.<sup>56</sup> Vectors were slowly released from this scaffold, yielding transfection *in vitro* comparable to bolus transfection controls. *In vivo* studies demonstrated a six- to eightfold enhancement in transfection relative to plasmid DNA delivery. Adsorption of PEI/DNA complexes to silica nanoparticles<sup>57, 58</sup> resulted in transgene expression *in vitro* comparable to that observed by bolus delivery and with reduced toxicity. Plasmid DNA has also been incorporated into inorganic calcium phosphate coprecipitates that are adsorbed onto PLGA matrices, which are mostly released by 48 h.<sup>59</sup>

### Virus Delivery

Viral vectors are more widely used than nonviral vectors due to their increased efficiency, yet their use requires further developments to make them less toxic and immunogenic.<sup>60</sup> Retroviruses and adenoviruses are the most widely used vectors in clinical trials, although other viruses are being used (herpes simplex, adeno-associated). Retroviruses offer the potential for long-term gene expression through integration into the host cell genome; however, obtaining efficient delivery, transducing nondividing cells, and achieving stable expression at an appropriate level are challenges that persist. Adenoviral vectors, on the other hand, can transduce a wide range of cells, including nondividing ones, and are relatively safe, though they can elicit a strong immune response. The stability of gene expression by adenoviral vectors may also be insufficient. Viruses can be engineered to be replication deficient, to reduce the immune response, and to target the virus to an appropriate cell type. Polymeric delivery may enhance delivery efficiency of viral vectors by overcoming some of the extracellular barriers.

Viral particles are composed of a nucleic acid genome surrounded by a capsid of proteins, which have the potential to interact with a polymeric delivery system. Most studies examining polymeric virus delivery have employed adenoviral vectors, which may provide a foundation for the use of other viral vector systems. Interactions with the material could provide some stability against degradation, as viruses can have half-lives on the order of hours at 37°C. This interaction between the virus and the polymer may also affect the immune response, which can target the vector or the transduced cells, thus decreasing transduction or the activity of the secreted protein.<sup>61–63</sup> Polymeric delivery of viral vectors may reduce recognition by the immune system by limiting binding of neutralizing antibodies and minimizing the amount of DNA necessary for gene transfer.<sup>64, 65</sup> En-

capsulated adenoviral vectors provided gene transfer in preimmunized mice,<sup>65, 66</sup> with encapsulated vectors inducing 45-fold lower anti-adenovirus titers than those obtained with direct injection.<sup>65, 67</sup>

Viruses can be encapsulated and released from both synthetic and natural materials, with the preparation procedure affecting both the fraction released and the activity. Incorporation of adenovirus into collagen resulted in release of 10 to 20% depending upon the collagen content.<sup>5, 68, 69</sup> *In vivo* application of collagen loaded with viral particles resulted in localized delivery and extended availability at the site of implantation. The viral particles retained their activity *in vitro* and *in vivo*, with expression at implantation sites *in vivo* lasting for months without redosing.<sup>5, 64, 65, 67, 68, 70</sup> Alternatively, collagen/alginate microspheres released 100% of the encapsulated virus within 48 h and were able to transduce cells *in vivo* at levels comparable to equivalent amounts of injected adenovirus.<sup>71</sup> Microspheres composed of PLG released 14 to 45% of encapsulated virus *in vitro*, depending on fabrication procedures.<sup>64, 65</sup> Virus was released for more than a week, with 70 to 90% released within the first 4 days<sup>64, 65</sup>; however, most of the viable virus was released during the first 2 days.<sup>65</sup>

Substrate-mediated delivery of viral vectors has been achieved through both specific and nonspecific binding of the virus to the polymer. Polystyrene beads and microspheres bind adenovirus vectors nonspecifically,

which increased transduction efficiency relative to free vector delivery and targeted gene expression in cells in contact with sphere *in vitro* and *in vivo*.<sup>72, 73</sup> Specific interactions of viral particles with the polymer have been incorporated through modification of the biomaterial or the virus with functional groups, such as antibodies or biotin residues. Collagen gels modified with antibodies to immobilize vectors localized transduction *in vivo* relative to control conditions.<sup>11, 74, 75</sup> Alternatively, adenovirus vectors have been chemically modified with biotin groups that are then bound to avidin-conjugated microspheres.<sup>76</sup> This approach transduced cells immediately adjacent to the beads *in vitro* and enhanced transgene expression for cells that are not readily transduced with adenovirus.<sup>76</sup> Recently, viruses have been engineered with functional groups in the viral shell, which would enable binding without chemical modification that can inactivate the virus.<sup>77-79</sup>

## Applications

Polymeric delivery of nonviral and viral vectors generally promotes gene transfer to cells within or adjacent to the implant. Depending on the choice of gene product, the protein produced by transfected cells may function either locally or systemically. Localized protein production is being used to stimulate an immune response, deliver a suicide gene, or promote wound healing. Alterna-

**Table 1.** *In vivo* studies involving therapeutically relevant genes delivered with polymeric systems.

Vector	Material	Species/location	Gene	Result	Ref
Plasmid	Collagen	Rat/femur	BMP-4/hPTH1-34	Bone formation	[88]
Plasmid	Collagen	Canine/bone	hPTH1-34	Bone formation over 1-cm gap	[18]
Plasmid	Collagen	Mice/intramuscular	FGF-4	Platelets increased for 60 days	[17]
Plasmid	PLG	Rat/subdermal	PDGF	Enhanced matrix deposition and vascularization	[7]
Adenovirus (FGF-2 target)	PVA/collagen	Rat/subdermal	PDGF	Granulation tissue	[69]
Canarypox virus	Gelatin	Mouse/intratympanic	IL-2, IL-12, TNF- $\alpha$	Growth inhibition of tumors	[86]
Plasmid	Collagen	Rabbit/ear	PDGF	Granulation tissue and epithelialization	[92]
Poly-D-lysine/plasmid	Collagen	Rat/optic nerve	FGF2, BDNF, NT-3	Survival of axotomized RGCs	[91]
Adenovirus	Collagen	Rabbit/ear	PDGF, FGF-2, VEGF	Granulation tissue deposition, wound closure, vascularization	[68]
Adenovirus/plasmid	Collagen	Rat/intramuscular	PDGF, FGF-2, FGF-6	Early angiogenesis, muscle repair	[93]
Plasmid	PLG	Mouse/subdermal	Endothelial locus-1	Small blood vessel formation	[34]
Plasmid	Gelatin	Mouse/renal subcapsule	MMP-1	Decreased blood urea nitrogen	[43]
Plasmid	Chitosan	Mouse/intranasal	RSV antigens	Reduction of viral titers and viral antigen load	[85]
Plasmid	EVAc	Mouse/intravaginal	LDH-C4	Induction of specific IgA	[36]
Plasmid	Gelatin	Rabbit/hindlimb	FGF-4	Angiogenesis	[101]
Adenovirus	Collagen	Mouse/dermal wound	PDGF-B	Granulation tissue and vascularization	[70]
Adenovirus	Collagen	Rabbit/dermal wound	PDGF-B	Granulation tissue, no toxicity	[70]

tively, protein secreted by locally transfected cells can be distributed systemically, which has applications to disorders such as hemophilia. The versatility of polymeric delivery to alter protein concentrations locally or systemically may impact numerous applications *in vivo* and *in vitro*. Table 1 lists studies that have employed polymeric delivery *in vivo* to induce physiological responses.

### Gene Therapy

Numerous clinical trials have been completed or are pending for a multitude of pathologies, including cancer (e.g., colorectal, bladder, and brain), monogenic disease, and vascular disease. Most trials have not shown significant therapeutic efficacy or clinically useful responses, likely due to inefficient delivery, lack of stable gene expression, and immune clearance of either the vector or the cells expressing a foreign gene.<sup>80-84</sup> Polymeric-based gene delivery systems may enhance delivery of the vector and extend the duration of transgene expression to achieve sufficient protein quantities that act locally or systemically. For example, intranasally delivered nanospheres loaded with plasmid encoding RSV antigens reduced viral titers and viral antigen load after acute RSV infection in mice.<sup>85</sup> Additionally, IL-2, IL-12, and TNF- $\alpha$  expression induced by a virus-releasing gelatin sponge inhibited tumor growth in heterotopic nodules of tumor-bearing mice.<sup>86</sup>

### Tissue Engineering

The challenge of tissue engineering lies in creating an environment that provides the appropriate combination of signals to induce proper cell function and restore normal tissue function. The scaffold functions as a support for cell growth and localized DNA delivery can provide the signals to direct progenitor cell differentiation. Although several fundamental requirements for the scaffold structure have been identified,<sup>87</sup> the design principles underlying gene delivery in tissue regeneration remain to be identified. Currently, a phase I clinical study using collagen-embedded adenovirus encoding PDGF has begun to evaluate the safety and maximum tolerated dose for treatment of diabetic ulcers.<sup>70</sup> Collagen-based delivery of nonviral or viral DNA has been employed in models of bone,<sup>18, 88, 89</sup> cartilage,<sup>90</sup> and nerve regeneration<sup>91</sup>; wound healing<sup>68-70, 92</sup>; muscle repair<sup>93</sup>; and cardiovascular disease.<sup>94</sup> Alternatively, viruses have been tethered to endovascular microcoils,<sup>74</sup> stents,<sup>75</sup> and heart valve replacement cusps<sup>95</sup> to localize delivery to the arterial wall and avoid spread to distal sites.<sup>75</sup> Porous PLG scaffolds releasing plasmid DNA were able to transfect cells within and around the scaffold, with sufficient expression of PDGF to promote tissue formation.<sup>7</sup> While these studies have illustrated the potential for extending the production of growth factors locally, adapting the delivery strategies to control transgene expression spatially (microme-

ters to millimeters) or temporally (days to months) may re-create the environmental complexity present during tissue formation.<sup>96</sup> The ability to regulate expression of one or more factors in time and space may be critical to the engineering of complex tissue architectures, such as those found in vascular networks and the nervous system. These systems would also increase our understanding of the biology behind tissue formation, which would serve to identify how gene delivery can best augment the regenerative process.

### Functional Genomics

Transfected cell arrays represent a high-throughput approach to correlate gene expression with functional cell responses, based on gene delivery from a surface.<sup>97</sup> In principle, this system can be employed for numerous studies, such as screening large collections of cDNAs<sup>97</sup> or targets for therapeutic intervention.<sup>98</sup> Transfected cell arrays were formed using a substrate-mediated approach in which plasmids or adenoviruses were mixed with collagen and spotted onto glass slides or into wells.<sup>97-99</sup> Plated cells were transfected and could be analyzed for cellular responses using a variety of imaging or biochemical techniques. Further development of the substrate-mediated approach requires the development of a cost-effective delivery system that efficiently transfects a wide variety of primary cells and cell lines, while allowing for spatially controlled DNA within the different domains.<sup>97, 100</sup>

### Conclusions

In comparison to traditional gene delivery systems, controlled release can enhance gene delivery by increasing the extent and duration of transgene expression, while reducing the need for multiple interventions. Additionally, localized vector delivery to specific tissues can avoid distribution to distant tissues, decrease toxicity to nontarget cells, and reduce the immune response. These polymer-based gene delivery systems capitalize on both specific and nonspecific interactions between the biomaterial and the vector, to achieve either release into the extracellular space or immobilization at the surface. While the potential to use these polymeric systems has been established, the design parameters by which to optimize or control gene transfer are not well understood. Vector and biomaterial development, combined with studies that correlate system properties (e.g., dose, release rate) with the quantity and duration of protein production and the number and location of cells expressing the transgene, will lead to molecular-scale design of delivery systems. The development of these systems may increase the efficacy within current gene therapy trials and may also extend the applicability of gene delivery to other areas such as tissue engineering and functional genomics.

## Acknowledgments

We thank Tatiana Segura, Zain Bengali, and Tiffany Houchin for their critical evaluation of the manuscript. This work was supported, in part, by grants from the NSF (BES0092701 [L.D.S.], Graduate Research Fellowship [A.K.P.]), the NIH (RO1GM066830), and the Christopher Reeve Paralysis Foundation.

## References

- Luo, D. and Saltzman, W. M. (2000). Enhancement of transfection by physical concentration of DNA at the cell surface. *Nat. Biotechnol.* 18: 893–895.
- Palsson, B. and Andreadis, S. (1997). The physico-chemical factors that govern retrovirus-mediated gene transfer. *Exp. Hematol.* 25: 94–102.
- Tam, P., *et al.* (2000). Stabilized plasmid-lipid particles for systemic gene therapy. *Gene Ther.* 7: 1867–1874.
- Layne, S. P., *et al.* (1992). Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. *Virology.* 189: 695–714.
- Ochiya, T., Nagahara, S., Sano, A., Itoh, H. and Terada, M. (2001). Biomaterials for gene delivery: Atelocollagen-mediated controlled release of molecular medicines. *Curr. Gene Ther.* 1: 31–52.
- Scherer, F., Schillinger, U., Putz, U., Stemberger, A. and Plank, C. (2002). Nonviral vector loaded collagen sponges for sustained gene delivery in vitro and in vivo. *J. Gene Med.* 4: 634–643.
- Shea, L. D., Smiley, E., Bonadio, J. and Mooney, D. J. (1999). DNA delivery from polymer matrices for tissue engineering. *Nat. Biotechnol.* 17: 551–554.
- Sakiyama-Elbert, S. E., Panitch, A. and Hubbell, J. A. (2001). Development of growth factor fusion proteins for cell-triggered drug delivery. *FASEB J.* 15: 1300–1302.
- Proctor, R. A. (1987). Fibronectin: A brief overview of its structure, function, and physiology. *Rev. Infect. Dis.* 9: (Suppl. 4): S317–S321.
- Bajaj, B., Lei, P. and Andreadis, S. T. (2001). High efficiencies of gene transfer with immobilized recombinant retrovirus: Kinetics and optimization. *Biotechnol. Prog.* 17: 587–596.
- Levy, R. J., *et al.* (2001). Localized adenovirus gene delivery using antiviral IgG complexation. *Gene Ther.* 8: 659–667.
- Segura, T. and Shea, L. D. (2002). Surface-tethered DNA complexes for enhanced gene delivery. *Bioconjugate Chem.* 13: 621–629.
- Putney, S. D. and Burke, P. A. (1998). Improving protein therapeutics with sustained-release formulations. *Nat. Biotechnol.* 16: 153–157.
- Norde, W. and Lyklema, J. (1991). Why proteins prefer interfaces. *J. Biomater. Sci. Polym. Ed.* 2: 183–202.
- Panyam, J. and Labhasetwar, V. (2003). Biodegradable nanoparticles for drug and gene delivery to cells and tissue. *Adv. Drug Delivery Rev.* 55: 329–347.
- Mathiowitz, E., *et al.* (1997). Biologically erodable microspheres as potential oral drug delivery systems. *Nature.* 386: 410–414.
- Ochiya, T., *et al.* (1999). New delivery system for plasmid DNA in vivo using atelocollagen as a carrier material: The Minipellet. *Nat. Med.* 5: 707–710.
- Bonadio, J., Smiley, E., Patil, P. and Goldstein, S. (1999). Localized, direct plasmid gene delivery in vivo: Prolonged therapy results in reproducible tissue regeneration. *Nat. Med.* 5: 753–759.
- Xie, Y., Yang, S. T. and Kniss, D. A. (2001). Three-dimensional cell-scaffold constructs promote efficient gene transfection: implications for cell-based gene therapy. *Tissue Eng.* 7: 585–598.
- Varde, N. K. and Pack, D. W. (2004). Microspheres for controlled release drug delivery. *Expert Opin. Biol. Ther.* 4: 35–51.
- Batzycki, R. P., Hanes, J., Langer, R. and Edwards, D. A. (1997). A theoretical model of erosion and macromolecular drug release from biodegrading microspheres. *J. Pharm. Sci.* 86: 1464–1477.
- Ando, S., Putnam, D., Pack, D. W. and Langer, R. (1999). PLGA microspheres containing plasmid DNA: Preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization. *J. Pharm. Sci.* 88: 126–130.
- Jang, J. H. and Shea, L. D. (2003). Controllable delivery of non-viral DNA from porous scaffolds. *J. Controlled Release.* 86: 157–168.
- Samuel, R. E., *et al.* (2002). Delivery of plasmid DNA to articular chondrocytes via novel collagen-glycosaminoglycan matrices. *Hum. Gene Ther.* 13: 791–802.
- Quick, D. J. and Anseth, K. S. (2003). Gene delivery in tissue engineering: A photopolymer platform to coencapsulate cells and plasmid DNA. *Pharm. Res.* 20: 1730–1737.
- Yun, Y. H., Goetz, D. J., Yellen, P. and Chen, W. (2004). Hyaluronan microspheres for sustained gene delivery and site-specific targeting. *Biomaterials.* 25: 147–157.
- Niidome, T. and Huang, L. (2002). Gene therapy progress and prospects: Nonviral vectors. *Gene Ther.* 9: 1647–1652.
- Nishikawa, M. and Huang, L. (2001). Nonviral vectors in the new millennium: Delivery barriers in gene transfer. *Hum. Gene Ther.* 12: 861–870.
- Herweijer, H. and Wolff, J. A. (2003). Progress and prospects: Naked DNA gene transfer and therapy. *Gene Ther.* 10: 453–458.
- Ledley, F. D. (1996). Pharmaceutical approach to somatic gene therapy. *Pharm. Res.* 13: 1595–1614.
- Zaharoff, D. A., Barr, R. C., Li, C. Y. and Yuan, F. (2002). Electromobility of plasmid DNA in tumor tissues during electric field-mediated gene delivery. *Gene Ther.* 9: 1286–1290.
- De Rosa, G., Quaglia, F., La Rotonda, M. I., Appel, M., Alphanthary, H. and Fattal, E. (2002). Poly(lactide-co-glycolide) microspheres for the controlled release of oligonucleotide/polyethylenimine complexes. *J. Pharm. Sci.* 91: 790–799.
- Kim, A., Checkla, D. M., Dehazya, P. and Chen, W. L. (2003). Characterization of DNA-hyaluronan matrix for sustained gene transfer. *J. Controlled Release.* 90: 81–95.
- Eliasz, R. E. and Szoka, F. C. Jr. (2002). Robust and prolonged gene expression from injectable polymeric implants. *Gene Ther.* 9: 1230–1237.
- Nof, M. and Shea, L. D. (2002). Drug-releasing scaffolds fabricated from drug-loaded microspheres. *J. Biomed. Mater. Res.* 59: 349–356.
- Shen, H., Goldberg, E. and Saltzman, W. M. (2003). Gene expression and mucosal immune responses after vaginal DNA immunization in mice using a controlled delivery matrix. *J. Controlled Release.* 86: 339–348.
- Jong, Y. S., *et al.* (1997). Controlled release of plasmid DNA. *J. Controlled Release.* 47: 123–134.
- Panyam, J., Zhou, W. Z., Prabha, S., Sahoo, S. K. and Labhasetwar, V. (2002). Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: Implications for drug and gene delivery. *FASEB J.* 16: 1217–1226.
- Sandor, M., *et al.* (2002). Transfection of HEK cells via DNA-loaded PLGA and P(FASA) nanospheres. *J. Drug Targeting.* 10: 497–506.
- Cohen, H., *et al.* (2000). Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. *Gene Ther.* 7: 1896–1905.
- Leong, K. W., Mao, H. Q., Truong-Le, V. L., Roy, K., Walsh, S. M. and August, J. T. (1998). DNA-polycation nanospheres as non-viral gene delivery vehicles. *J. Controlled Release.* 53: 183–193.
- Truong-Le, V. L., *et al.* (1999). Gene transfer by DNA-gelatin nanospheres. *Arch. Biochem. Biophys.* 361: 47–56.
- Aoyama, T., Yamamoto, S., Kanematsu, A., Ogawa, O. and Tabata, Y. (2003). Local delivery of matrix metalloproteinase gene prevents the onset of renal sclerosis in streptozotocin-induced diabetic mice. *Tissue Eng.* 9: 1289–1299.
- Zheng, J., Manuel, W. S. and Hornsby, P. J. (2000). Transfection of cells mediated by biodegradable polymer materials with surface-bound polyethyleneimine. *Biotechnol. Prog.* 16: 254–257.
- Cohen-Sacks, H., *et al.* (2004). Delivery and expression of pDNA embedded in collagen matrices. *J. Controlled Release.* 95: 309–320.
- Denis-Mize, K. S., *et al.* (2000). Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells. *Gene Ther.* 7: 2105–2112.
- Segura, T. and Shea, L. D. (2001). Materials for non-viral gene delivery. *Annu. Rev. Mater. Sci.* 31: 25–36.
- Varga, C. M., Hong, K. and Lauffenburger, D. A. (2001). Quantitative analysis of synthetic gene delivery vector design properties. *Mol. Ther.* 4: 438–446.
- Capan, Y., Woo, B. H., Gebrekidan, S., Ahmed, S. and DeLuca, P. P. (1999). Stability of poly(L-lysine)-complexed plasmid DNA during mechanical stress and DNase I treatment. *Pharm. Dev. Technol.* 4: 491–498.
- Capan, Y., Woo, B. H., Gebrekidan, S., Ahmed, S. and DeLuca, P. P. (1999). Influence of formulation parameters on the characteristics of poly(DL-lactide-co-glycolide) microspheres containing poly(L-lysine) complexed plasmid DNA. *J. Controlled Release.* 60: 279–286.
- Huang, Y. C., Connell, M., Park, Y., Mooney, D. J. and Rice, K. G. (2003). Fabrication and in vitro testing of polymeric delivery system for condensed DNA. *J. Biomed. Mater. Res.* 67A: 1384–1392.

52. Moret, I., *et al.* (2001). Stability of PEI-DNA and DOTAP-DNA complexes: Effect of alkaline pH, heparin and serum. *J. Controlled Release.* 76: 169-181.
53. Zelphati, O., Uyechi, L. S., Barron, L. G. and Szoka, F. C. Jr. (1998). Effect of serum components on the physico-chemical properties of cationic lipid/oligonucleotide complexes and on their interactions with cells. *Biochim. Biophys. Acta.* 1390: 119-133.
54. De Rosa, G., Quaglia, F., La Rotonda, M. I., Besnard, M. and Fattal, E. (2002). Biodegradable microparticles for the controlled delivery of oligonucleotides. *Int. J. Pharm.* 242: 225-228.
55. Segura, T., Volk, M. J. and Shea, L. D. (2003). Substrate-mediated DNA delivery: Role of the cationic polymer structure and extent of modification. *J. Controlled Release.* 93: 69-84.
56. Bielinska, A. U., *et al.* (2000). Application of membrane-based dendrimer/DNA complexes for solid phase transfection in vitro and in vivo. *Biomaterials.* 21: 877-887.
57. Kneuer, C., *et al.* (2000). A nonviral DNA delivery system based on surface modified silica-nanoparticles can efficiently transfect cells in vitro. *Bioconjugate Chem.* 11: 926-932.
58. Manuel, W. S., Zheng, J. I. and Hornsby, P. J. (2001). Transfection by polyethyleneimine-coated microspheres. *J. Drug Targeting.* 9: 15-22.
59. Kofron, M. D. and Laurencin, C. T. (2004). Development of a calcium phosphate co-precipitate/poly(lactide-co-glycolide) DNA delivery system: Release kinetics and cellular transfection studies. *Biomaterials.* 25: 2637-2643.
60. Anderson, W. F. (1998). Human gene therapy. *Nature.* 392: 25-30. |
61. Alden, T. D., *et al.* (1999). In vivo endochondral bone formation using a bone morphogenetic protein 2 adenoviral vector. *Hum. Gene Ther.* 10: 2245-2253.
62. Musgrave, D. S., Bosch, P., Ghivizzani, S., Robbins, P. D., Evans, C. H. and Huard, J. (1999). Adenovirus-mediated direct gene therapy with bone morphogenetic protein-2 produces bone. *Bone.* 24: 541-547.
63. Okubo, Y., Bessho, K., Fujimura, K., Iizuka, T. and Miyatake, S. I. (2000). Osteoinduction by bone morphogenetic protein-2 via adenoviral vector under transient immunosuppression. *Biochem. Biophys. Res. Commun.* 267: 382-387.
64. Beer, S. J., Hilfinger, J. M. and Davidson, B. L. (1997). Extended release of adenovirus from polymer microspheres: Potential use in gene therapy for brain tumors. *Adv. Drug Delivery Rev.* 27: 59-66.
65. Beer, S. J., Matthews, C. B., Stein, C. S., Ross, B. D., Hilfinger, J. M. and Davidson, B. L. (1998). Poly (lactic-glycolic) acid copolymer encapsulation of recombinant adenovirus reduces immunogenicity in vivo. *Gene Ther.* 5: 740-746.
66. Sailaja, G., HogenEsch, H., North, A., Hays, J. and Mittal, S. K. (2002). Encapsulation of recombinant adenovirus into alginate microspheres circumvents vector-specific immune response. *Gene Ther.* 9: 1722-1729.
67. Matthews, C., Jenkins, G., Hilfinger, J. and Davidson, B. (1999). Poly-L-lysine improves gene transfer with adenovirus formulated in PLGA microspheres. *Gene Ther.* 6: 1558-1564.
68. Doukas, J., *et al.* (2001). Matrix immobilization enhances the tissue repair activity of growth factor gene therapy vectors. *Hum. Gene Ther.* 12: 783-798.
69. Chandler, L. A., *et al.* (2000). FGF2-targeted adenovirus encoding platelet-derived growth factor-B enhances de novo tissue formation. *Mol. Ther.* 2: 153-160.
70. Gu, D., Nguyen, T., Phillips, M. L., Chandler, L. A. and Sosnowski, B. A. (2004). Matrix-immobilized growth factor gene therapy enhances tissue repair. *Wounds.* 16: 34-41.
71. Kalyanasundaram, S., Feinstein, S., Nicholson, J. P., Leong, K. W. and Garver, R. I. Jr. (1999). Coacervate microspheres as carriers of recombinant adenoviruses. *Cancer Gene Ther.* 6: 107-112.
72. Cavanagh, H. M., Dingwall, D., Steel, J., Benson, J. and Burton, M. (2001). Cell contact dependent extended release of adenovirus by microparticles in vitro. *J. Virol. Methods.* 95: 57-64.
73. Mah, C., *et al.* (2002). Improved method of recombinant AAV2 delivery for systemic targeted gene therapy. *Mol. Ther.* 6: 106-112.
74. Abrahams, J. M., Song, C., DeFelice, S., Grady, M. S., Diamond, S. L. and Levy, R. J. (2002). Endovascular microcoil gene delivery using immobilized anti-adenovirus antibody for vector tethering. *Stroke.* 33: 1376-1382.
75. Klugherz, B. D., *et al.* (2002). Gene delivery to pig coronary arteries from stents carrying antibody-tethered adenovirus. *Hum. Gene Ther.* 13: 443-454.
76. Pandori, M., Hobson, D. and Sano, T. (2002). Adenovirus-microbead conjugates possess enhanced infectivity: A new strategy for localized gene delivery. *Virology.* 299: 204-212.
77. Parrott, M. B. and Barry, M. A. (2000). Metabolic biotinylation of recombinant proteins in mammalian cells and in mice. *Mol. Ther.* 1: 96-104.
78. Parrott, M. B. and Barry, M. A. (2001). Metabolic biotinylation of secreted and cell surface proteins from mammalian cells. *Biochem. Biophys. Res. Commun.* 281: 993-1000.
79. Lee, J. H., *et al.* (1999). Engineering novel cell surface receptors for virus-mediated gene transfer. *J. Biol. Chem.* 274: 21878-21884.
80. Pagliaro, L. C., *et al.* (2003). Repeated intravesical instillations of an adenoviral vector in patients with locally advanced bladder cancer: A phase I study of p53 gene therapy. *J. Clin. Oncol.* 21: 2247-2253.
81. Palmer, D. H., Chen, M. J. and Kerr, D. J. (2003). Taking gene therapy into the clinic. *J. Biomed. Biotechnol.* 2003: 71-77. |
82. Kerr, D. (2003). Clinical development of gene therapy for colorectal cancer. *Nat. Rev. Cancer.* 3: 615-622.
83. Rainovy, N. G. and Ren, H. (2003). Gene therapy for human malignant brain tumors. *Cancer J.* 9: 180-188.
84. Powell, J. S., *et al.* (2003). Phase 1 trial of FVIII gene transfer for severe hemophilia A using a retroviral construct administered by peripheral intravenous infusion. *Blood.* 102: 2038-2045.
85. Mohapatra, S. S. (2003). Mucosal gene expression vaccine: A novel vaccine strategy for respiratory syncytial virus. *Pediatr. Infect. Dis. J.* 22: S100-S103, discussion S103-104.
86. Siemens, D. R., Austin, J. C., Hedicani, S. P., Tartaglia, J. and Ratliff, T. L. (2000). Viral vector delivery in solid-state vehicles: Gene expression in a murine prostate cancer model. *J. Natl. Cancer Inst.* 92: 403-412.
87. Murphy, W. L. and Mooney, D. J. (1999). Controlled delivery of inductive proteins, plasmid DNA and cells from tissue engineering matrices. *J. Periodont. Res.* 34: 413-419.
88. Fang, J., *et al.* (1996). Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. *Proc. Natl. Acad. Sci. USA.* 93: 5753-5758.
89. Labhasetwar, V., Bonadio, J., Goldstein, S. A. and Levy, J. R. (1999). Gene transfection using biodegradable nanospheres: Results in tissue culture and a rat osteotomy model. *Colloids Surf. B.* 16: 281-290.
90. Pascher, A., *et al.* (2004). Gene delivery to cartilage defects using coagulated bone marrow aspirate. *Gene Ther.* 11: 133-141.
91. Berry, M., *et al.* (2001). Sustained effects of gene-activated matrices after CNS injury. *Mol. Cell. Neurosci.* 17: 706-716.
92. Tyrone, J. W., *et al.* (2000). Collagen-embedded platelet-derived growth factor DNA plasmid promotes wound healing in a dermal ulcer model. *J. Surg. Res.* 93: 230-236.
93. Doukas, J., *et al.* (2002). Delivery of FGF genes to wound repair cells enhances arteriogenesis and myogenesis in skeletal muscle. *Mol. Ther.* 5: 517-527.
94. Klugherz, B. D., *et al.* (2000). Gene delivery from a DNA controlled-release stent in porcine coronary arteries. *Nat. Biotechnol.* 18: 1181-1184.
95. Stachelek, S. J., *et al.* (2004). Localized gene delivery using antibody tethered adenovirus from polyurethane heart valve cusps and intra-aortic implants. *Gene Ther.* 11: 15-24.
96. Saltzman, W. M. and Olbricht, W. L. (2002). Building drug delivery into tissue engineering. *Nat. Rev. Drug Discovery.* 1: 177-186.
97. Ziauddin, J. and Sabatini, D. M. (2001). Microarrays of cells expressing defined cDNAs. *Nature.* 411: 107-110.
98. Michiels, F., *et al.* (2002). Arrayed adenoviral expression libraries for functional screening. *Nat. Biotechnol.* 20: 1154-1157.
99. Honma, K., *et al.* (2001). Atelocollagen-based gene transfer in cells allows high-throughput screening of gene functions. *Biochem. Biophys. Res. Commun.* 289: 1075-1081.
100. Wu, R. Z., Bailey, S. N. and Sabatini, D. M. (2002). Cell-biological applications of transfected-cell microarrays. *Trends Cell Biol.* 12: 485-488.
101. Kasahara, H., *et al.* (2003). Biodegradable gelatin hydrogel potentiates the angiogenic effect of fibroblast growth factor 4 plasmid in rabbit hindlimb ischemia. *J. Am. Coll. Cardiol.* 41: 1056-1062.