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Delivery of a DNAzyme targeting *c-myc* to HT29 colon carcinoma cells using a gold nanoparticulate approach

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The objective of the current study was to develop cellular delivery approaches for catalytic DNA enzymes (DNAzymes) which cleave targeted messenger RNA, using vectors based on colloidal gold. The model DNAzyme was a 32mer oligonucleotide designed to specifically interact with and cleave c-myc mRNA. Colloidal gold particles were prepared by reduction of tetrachlororauric [III] acid with sodium citrate. Particles could be produced in the 1–90 nm range. A cationic substrate linked to transferrin was electrostatically/hydrophobically bound to the gold particle. These vectors were then treated with the DNAzyme to yield the condensed DNA-cationic polymer-particulate product. The pH (4–11.5), the quantity of the DNAzymes (0.079–0.567 μg/probe), the cationic polymer (polylysine (PL) or polyethylenimine (PEI)) as well as the surfactant (PVP) concentration (0–0.5 %) were varied to give stable constructs which decomplexed under the desired conditions (i.e., in lysosomes and at lower pH values). Cellular uptake of the FITC-labelled c-myc DNAzyme incorporated in this vector was measured using FACS analysis in human HT29 colon carcinoma cells. Data suggested that PEI gave better delivery efficiencies than PL. The use of PVP to stabilize the formed dispersions was detrimental to DNAzyme delivery when PL was used but had little effect in the PEI systems. In the best cases, delivery to 77% of the cells was possible using PEI with the PVP stabilizer and completing the DNA condensation at pH 5.5 with 0.118 μg of DNAzyme/probe. In contrast, the best conditions for PL gave only transfection to 43% of the cells (no PVP, condensed at pH 5.7 and with a loading of 0.079 μg DNAzyme/probe). The PL probe tended to be more toxic than the PEI-based systems (65% cell death in PL transfected cells compared to 22% for PEI). These results suggest that cellular targeting using colloidal gold appears feasible for DNAzyme delivery.

1. Introduction

DNAzymes are synthetic, catalytic deoxyribonucleic acid derivatives that can be engineered to bind to specific complementary RNA targets through Watson-Crick base pairing (Cairnes et al. 1999; Khachigian 2002). These DNA fragments can then cleave the targeted RNA at a predefined position resulting in gene down regulation and suppression or elimination in gene product production. DNAzymes have a number of advantages over ribozyme (the analogous RNA constructs)-based delivery, derived mainly from their greater stability to nucleolytic degradation. An example of a DNAzyme is given in Fig. 1. A number of gene products have been targeted with DNAzymes including those for HIV-1 env, HIV-1 tat/rev, CCR5, BCR-ABL, PKCα, Egr1, c-myc, k-ras and c-jun making these therapeutics applicable to cancer, antiviral and restenosis indications (Sun et al. 1999; Sun and Cairns 2000; Achenbach et al. 2004; Sioud and Iversen 2005; Kelley and Patterson 2006; Isaka 2007).

For a DNAzyme to be active, it must reach its site of action within the cells meaning it must penetrate the cell membrane, be appropriately trafficked in the cells and reach the nucleus. The challenges associated with the delivery of DNA or RNA-derived materials are numerous as has been pointed out both in the scientific literature and in the popular press: "The weak link of gene therapy is paradoxically the vehicle rather than the drug itself" (Behr 1994), "The key to success for any gene therapy strategy is having a vector able to serve as a safe and efficient gene delivery vehicle" (Friedmann 1997), "There are only three problems in gene delivery: delivery, delivery and delivery" (Jaroff 1999).

A number of methods have been brought to bear on this problem with varying degrees of success. Pun et al. (2004) described the use of cationic polymers based on cyclodextrins to deliver functional DNAzymes to cells and, *in vivo*, to tumors in mice. Other delivery approaches including cationic liposomes and dendrimers as well as peptide-based

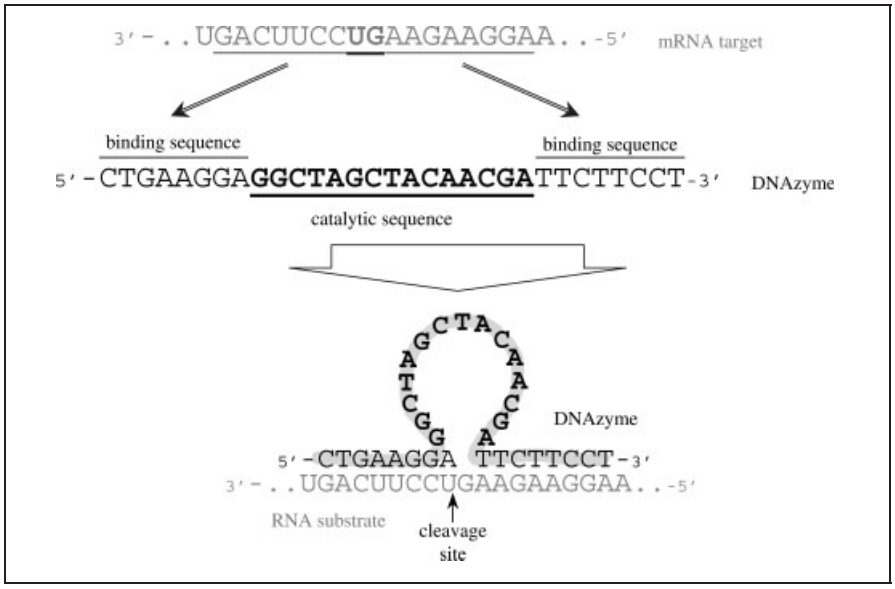

Fig. 1:
General structure of DNAzyme and their mechanism of action. Upon recognition and binding to the target mRNA, the DNAzyme takes the shape of a hammerhead ribozyme having two binding arms of about 8 nucleotides each specific to the given RNA substrate and a catalytic loop made up of about 15 nucleotides that chelates catalytic metal ions such as Mg^{++}. The recognition arms bind the targeted RNA via Watson-Crick based pairing and the catalytic domain of the DNAzyme can cleave a phosphodiester linkage located between a purine and pyrimidine residue

vectors have also been suggested for the delivery of antisense RNA and other genetic materials (Dass et al. 2002; Tack et al. 2006a, c). Finally, several researchers have suggested the use of colloidal metals for delivering cytokines, growth factors, chemotherapeutic agents and nucleic acids to cells of interest (Tamarkin and Paciotti 1999, 2002, O'Sullivan et al. 2003; Noh et al. 2007).
The current study was aimed at assessing the ability to deliver a model DNAzyme to cells using colloidal gold probes. Specifically, a DNAzyme based on *c-myc* was chosen with delivery to HT29 cells, a human colon carcinoma cell line, selected as the initial proof-of-concept endpoint.

2. Investigations, results and discussion

Colloidal gold can be produced by reacting gold acids with various reducing agents (Oliver 1999; Schmid and Corain 2003; Daniel and Astruc 2004; Haiss et al. 2007). The conditions and reducing agents used can vary the particle size of the dispersion. Gold particles of 10 nm were selected since larger particles can sediment over time and smaller particles may not be adequately covered by the protein carriers of interest.
Coating of gold particles with methylated albumin was possible at pH 8 and 10 with about 40 μg being optimal to coat 1 mL of a 10 nm gold dispersion (as described in the Experimental Section). The particles were stable to NaCl-induced aggregation at pH values between 6 and 12 (Fig. 2). Histone coating was possible at pH 12 with 15 μg of carrier per mL of probe optimal. The systems was stable to aggregation between pH 11 and 13. Poly-L-lysine could be coated on gold at a pH of 10 at an optimal amount of 29 μg/1 mL of dispersion. These probes were stable between pH 11 and 13.

Based on screening experiments that suggested little or no uptake of the gold probes either as such or coated with a carrier or carrier-DNAzyme, targetors were added to the probes. These targetors were designed to interact with cell-based proteins to facilitate internalization of the probe via receptor-mediated endocytosis or related processes. Based on preliminary studies, the optimal carrier was a conjugate between polylysine or polyethylenimine and transferrin. These data are consistent with the observation that transferrin receptors are commonly expressed on tumor cells and can aid in drug delivery (Qian et al. 2002; Daniels et al. 2006; Kobayashi et al. 2007) and that polyethylenimine and polylysine are widely used as successful transfecting agents (Zauner et al. 1998; Ruponen et al. 2003; Wagner and Kloeckner 2006).
Optimal binding of the PL-Tf or PEI-Tf conjugate to the DNAzyme was completed by analyzing various charge ratios of the two components on agarose gel and then looking for migration in an electric field (Noh et al. 2007). Optimal ratios can be identified at the point when excess DNAzyme just starts to migrate on the gel (Fig. 3).
Optimal DNAzyme binding to the gold-polymer-Tf probe was assessed using a spectrophotometric procedure. At a particular pH, the DNAzyme was added to a fixed concentration of the carrier-gold nanoprobe. The samples were centrifuged and the amount of DNAzyme unbound in the supernatant was measured by UV spectrophotometry at 260 nm. Maximum binding was indicated by the plateau and varied as a function of conditions. Figure 4 gives an example at pH 9 for the binding of gold adsorbed PEI-Tf and the DNAzyme. A schematic representation of the gold probe is given in Fig. 5.
Based on these preliminary experiments, a matrix was set up in which various probes were prepared and their stabi-

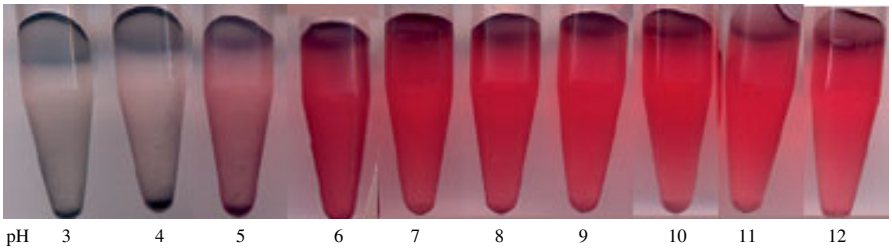

Fig. 2:
Colloid stability of 10 nm gold particles coated with methylated albumin at various pH values when treated with 100 μL of a 10% NaCl solution

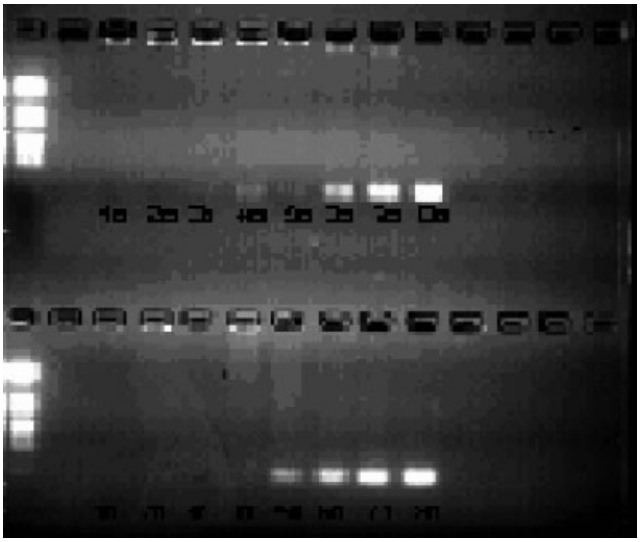

Fig. 3: Gel electrophoresis of various mixtures of FITC-labeled PL-Tf (upper panel) or FITC-labeled PEI-Tf (lower panel) and DNAzyme. Optimal ratios were obtained at approximately 2 µg DNAzyme/µg PL-Tf and 4–7 µg DNAzyme/µg PEI-Tf

lity assessed. In addition to what was done in previous experiments, polyvinyl pyrrolidone (0.5% w/v) was added as a co-stabilizer in some cases. These probes were then incubated with HT29 cells (a transferrin-receptor expressing cell line derived from a human colon carcinoma) and transfection measured using FACS cell sorting. The conditions and results are given in the Table.

The data suggested that the PEI-Tf conjugate gave better transfections than the analogous PL-Tf carrier. In the case of PEI-Tf, optimal delivery was possible by loading the carriers at either high or low pH, at high DNAzyme loading and in the presence of the PVP stabilizer. Total transfection in these cases were 73% (high pH) and 77% (low pH). Elimination of the stabilizer reduced transfection to 53% (high pH) and 56% (low pH). Interestingly, the probe prepared at low pH tended to be less toxic to the cells with 60% of cell transfected surviving while the analogous probes prepared at high pH generated a live cell transfection value of 28%. The polylysine based probes were less efficient and more toxic than the PEI-based probes. The best transfections were obtained with gold particles loaded at low pH and with low DNAzyme concentrations and in the absence of the PVP stabilizer. Best delivery rates were 56% (total transfection) and 36% if only live cells were counted.

In conclusions, colloidal gold-based delivery of DNAzyme is possible through the use of cellular targetors such as transferrin. Optimal gold probes were prepared by adsorbing cationic polymer (PEI or PL)-transferrin conjugates onto the nanodispersion followed by condensation with the DNAzymes. Optimal transfections of >75% were possible into HT29 cells.

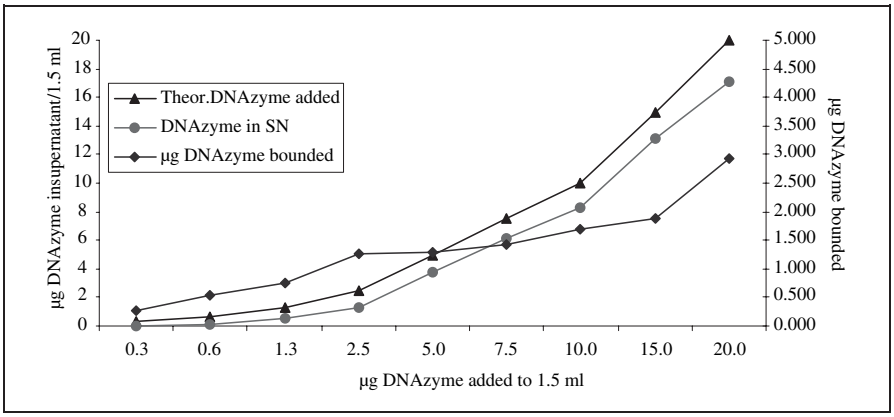

Fig. 4:
Titration curve of DNAzyme binding to a gold-PEI-Tf vector at pH 9. The amount of DNAzyme bound was determined by the amount of DNAzyme unbound in the supernatant subtracted by the total amount added

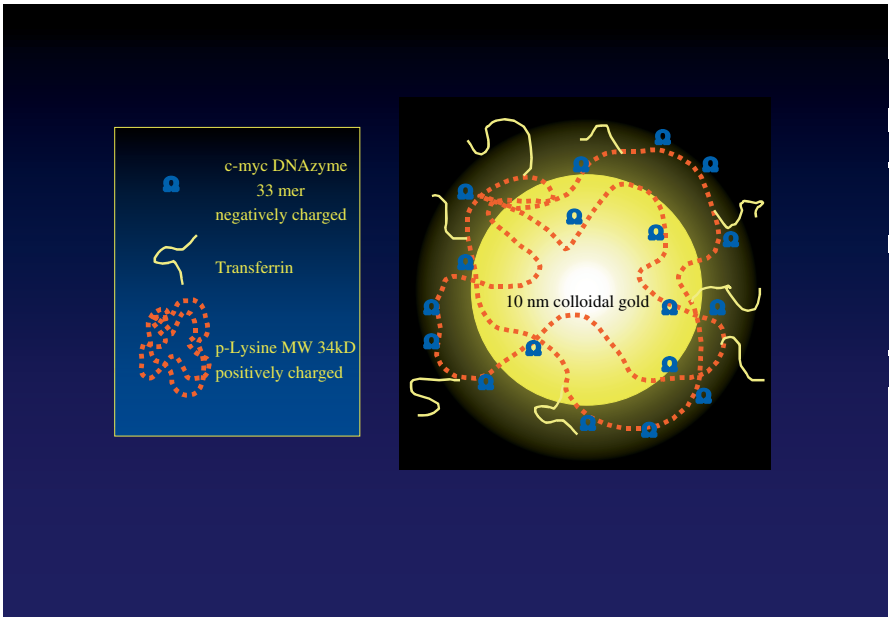

Fig. 5:
Schematic representation of a gold probe-based delivery system for a DNAzyme. In this instance, polylysine-transferrin served as the carrier

Table: Conditions for probe preparation and transfection efficiency for a DNAzyme into HT29 cells

Exp. No.	pH	μg DNAzyme/ μL probe	Conjugate	PVP 0.5% Y/N	% Total transfection	% Live cells transfected
1	11.15	0.079	PL-Tf	N	–	–
2	11.48	0.124	PEI-Tf	N	53	36
3	11.20	0.075	PL-Tf	Y	15	1
4	11.40	0.118	PEI-Tf	Y	73	28
5	5.70	0.079	PL-Tf	N	43	28
6	5.75	0.124	PEI-Tf	N	56	36
7	5.80	0.075	PL-Tf	Y	25	11
8	5.50	0.118	PEI-Tf	Y	77	60

3. Experimental

3.1. Materials

A DNAzyme targeted towards *c-myc* has been described by Sun et al. (1999). The DNAzyme as well as various labeled analogs (i.e., 5′-fluorescein labelled DNAzyme) (TGAGGGGCAGGCTAGCTACAACGAGTCCGCGGx-3′ where x = 3′dG5′) were obtained from Eurogentec (Seraing, Belgium). Stability was improved by the incorporation of a 3′dG5′-3′ thymidylate inversion at the 3′ end. Poly-L-lysine (MW = 10000 g/mol), histone Type VII-S and methylated albumin were obtained from Sigma Chemical Co., St. Louis, MO, USA. The polyethylenime-transferrin and polylysine-transferrin conjugate was obtained from Bender MedSystems, Vienna, Austria.

3.2. Preparation of colloidal gold

The preparation of the colloidal gold in the 10 nm range was accomplished by adding 1.7 mL of a 10% solution of $HAuCl_4$ to 24 mL of a 5% solution of sodium citrate. The colloidal gold dispersion was wine red in color. Solutions were prepared with Milli Q water which was boiled and then cooled with a stream of N_2 passing through it.

3.3. Carrier/Gold interaction studies

To select a cationic carrier and to identify the optimal conditions for attaching the carrier to the colloidal gold, the following procedure was used: 1 mL of the colloidal gold dispersion prepared above was used in these studies. The solution was adjusted to different pH values between 3 and 12 using 0.01 N NaOH. A fixed amount of the carrier of interest was then added to the solution. After several minutes, 100 μL of a 10% NaCl solution was added to the dispersion. The red solutions turned light blue and then precipitated after a few seconds if a sterically stabilized suspension was not formed by the added cationic carriers.

3.4. Carrier/DNAzyme interaction studies

The optimal binding conditions for the carrier and DNAzyme (in the absence of the colloidal gold) were completed using agarose electrophoresis. A fixed amount of the FITC labeled DNAzyme is added at varying concentrations of the carrier after which the solution is applied to an agarose gel. In conditions where there is no binding between the DNAzyme and carrier, the fluorescently labeled DNAzyme migrates with the applied electric current (2 h, 50 V). Binding of the two components neutralizes the DNAzyme charge resulting in a fluorescent spot which stays at the origin.

3.5. DNAzyme/carrier/gold interactions studies

Optimization of binding of the DNAzyme to the gold-carrier as a function of pH is completed using a spectrophotometric method. Solutions of the colloidal gold and carrier are adjusted to different pH values. Different amounts of the labeled DNAzyme are then added the solution centrifuged. The optical density of the supernatant (at 260 nm) is then assessed. Optimal binding is determined at the pH and inflection point where additional DNAzyme does not increase the absorption in the centrifuged solution.

3.6. DNAzyme binding assessment

To confirm that the DNAzyme is attached to the gold particle, a Zetaprobe Binding Assay is applied. A strand of DNA complementary to the DNAzyme is spotted onto Zetaprobe Nitrocellulose Membranes (Bio-Rad, Richmond, CA) and then cross-linked to the substrate using UV light. The DNAzyme/carrier/gold probe is then applied to the substrate at different pH's and allowed to stand overnight. The nitrocellulose substrate is then washed. If the DNAzyme is bound to the gold and to the complementary DNA substrate, a red spot will develop.

3.7. Illustrative preparation method

500 μL of an aqueous solution of PEI-Tf (0.12 μg/μL) was added to 2500 μL of a 10 nm colloidal gold dispersion adjusted to pH 11.6. The FITC-labelled DNAzyme (198 μL of a 2 mg/mL aqueous solution) was then added as was 160 μL of a 10% PVP solution (pH 9). This gave a probe in which 7 μg of the DNAzyme bound to 1 μg of the PEI-Tf carrier. The final concentration of PVP was 0.5%. 100 μL of the probe were then adjusted to pH 5.8 by the addition of 7.5 μL of 0.1 N HCl and 1 μl 0.01 N HCl.

3.8. Cell culture examination

HT29 cells, a human colon carcinoma cell line, were seeded at a concentration of 300,000 cell/well in 6-well culture dishes and maintained in McCoys 5A media until they reached 50–80% confluence. Cells were then washed with the media which was then replaced with the transfection media which contained the DNAzyme probes of interest. After 15 min, an additional aliquot of culture media was added and the cells were allowed to incubate for 24 h at 37 °C. The cells were then collected after trypsinization and resuspended in 1 mL of cold PBS. Propidium iodide as then added to a final concentration of 2 μg/mL and the cells analysed by FACS sorting (MoFlo, Cytomation).

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References

Achenbach J, Chiuman W, Cruz R, Li Y (2004) DNAzymes: from creation in vitro to applications in vivo. Curr Pharmaceut Biotech 5: 321–336.
Behr J (1994) Gene transfer with synthetic cationic amphiphiles: Prospects for gene delivery. Bioconjugate Chem. 5: 389–389.
Cairns M, Hopkins T, Witherington C, Wang L, Sun L (1999) Target site selection for an RNA-cleaving catalytic DNA. Nature Biotech 17: 480–486.
Daniel M, Astruc D (2004) Gold nanoparticles: assembly, supermolecular chemistry, quantum-size-related properties and applications towards biology, catalysis and nanotechnology. Chem Rev 104: 293–346.
Daniels T, Delgado T, Helguera G, Penichet M (2006) The transferring receptor part II: Targeted delivery of therapeutic agents into cancer cells. Clin Immunol 121: 159–176.
Dass C, Saravolac E, Li Y, Sun L (2002) Cellular uptake, distribution and stability of 10–23 deoxyribozymes. Antisense Nucleic Acid Drug Dev 12: 289–299.
Friedmann T (1997) Overcoming the obstacles to gene delivery. Scientific American June.
Haiss W, Thanh N, Aveyard J, Fernig D (2007) Determination of size and concentration of gold nanoparticles from UV-Vis spectra. Anal Chem 79: 4215–4221.
Isaka Y (2007) DNAzymes as potential therapeutic molecules. Curr Opin Molec Ther 9: 132–136.
Jaroff L (1999) Fixing the genes. Time Magazine Jan. 11.
Kelly R, Patterson C (2006) Slice me nice: DNAzyme get close to the heart. J Thromb Haemost 4: 1476–1478.
Khachigian L (2002) DNAzymes: cutting a path to a new class of therapeutics. Curr Opin Molec Therap 4: 119–121.
Kobayashi T, Ishida T, Okada Y, Ise S, Harashima H, Kiwada H (2007) Effect of transferring receptor-targeted liposomal doxorubicin in P-glycoprotein-mediated drug resistant tumor cells. Int J Pharm 329: 94–102.
Noh S, Kim W, Kim J, Baek K, Oh Y (2007) Enhanced cellular delivery and transfection efficiency of plasmid DNA using positively charged biocompatible colloidal gold nanoparticles. Biochim Biophys Acta 1770: 747–752.
Oliver C (1999) Preparation of colloidal gold. Method Molec Biol 115: 327–330.
O'Sullivan M, Green J, Przybycien T (2003) Development of a novel gene delivery scaffold utilizing colloidal gold-polyethylenimine conjugates for DNA condensation. Gene Ther 10: 1882–1890.
Pun S, Bellocq N, Cheng J, Grubbs B, Jensen G, Davis M, Tack F, Brewster M, Janicot M, Janssens B, Floren W, Bakker A (2004) Biodistribution of RNA-cleaving DNA enzyme (DNAzyme) to tumor tissue by transferrin-modified, cyclodextrin-based particles. Cancer Biol Ther 3: 641–650.

Qian Z, Li H, Sun H, Ho K (2002) Targeted drug delivery via the transferring receptor-mediated endocytosis pathway. Pharmacol Rev 54: 561–587.

Ruponen M, Honkakoski P, Ronkko S, Pelkonen J, Tammi M, Urtti A (2003) Extracellular and intracellular barriers to non-viral gene delivery. J Control Release 93: 213–217.

Schmid G, Corain B (2003) Nanoparticulated gold: synthesis, electronics and reactivities. Eur J Inorg Chem 3089–3098.

Souid M, Iversen P (2005) Ribozymes, DNAzymes and small interfering RNAs as therapeutics. Curr Drug Targets 6: 647–653.

Sun L, Cairns M, Gerlach W, Witherington C, Wang L, King A (1999) Supression of smooth muscle cell proliferation by a c-myc RNA-cleaving deoxyribozyme. J Biol Chem 274: 17236–17241.

Sun L, Cairns M (2000) Design of DNAzymes that specifically target c-myc mRNA and their use for treating restenosis. World Patent WO 2000009672.

Tack F, Bakker A, Maes S, Dekeyser N, Bruining M, Elissen-Roman C, Janicot M, Brewster M, Janssen H, De Waal B, Franssen P, Lou X, Meijer E (2006a) Modified poly(propylene imine) dendrimers as effective transfection agents for catalytic DNA enzymes (DNAzymes). J Drug Target 14: 69–86.

Tack F, Bakker A, Maes S, Dekeyser N, Brusining M, Elissen-Roman C, Janicot M, Hanssen H, De Waal B, Fransen P, Lou X, Meijer W, Arien T, Brewster M (2006b) Dendrimeric poly(propylene-imines) as effective delivery agents for DNAzymes: toxicity, in vitro transfection and in vivo delivery. J Control Release 116: E26-E28.

Tack F. Bakker A, Maes S, Dekeyser N, Brusining M, Elissen-Roman C, Janicot M, Hanssen H, De Waal B, Fransen P, Lou X, Meijer W, Arien T, Brewster M (2006c) Dendrimeric poly(propylene-iImines) as effective delivery agents for DNAzymes: Dendrimer synthesis, stability and oligonucleotide complexation. J Control Release 116: E24–E26.

Tamarkin L, Paciotti G (1999) Compositions and methods for targeted delivery of endogenous and exogenous bioactive agents. World Patent WO9924077.

Tamarkin L, Paciotti G (2002) Colloidal metal compositions and methods for drug targeting. World Patent WO2002087509.

Wagner E, Kloeckner J (2006) Gene delivery using polymer therapeutics. Adv Polymer Sci 192: 135–173.

Zauner W, Ogris M, Wagner E (1998) Polylysine-based transfection systems utilizing receptor-based delivery. Adv Drug Deliv Rev 30: 97–113.