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Characterization and performance of nucleic acid nanoparticles combined with protamine and gold

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ABSTRACT

Macromolecular nucleic acids such as DNA vaccines, siRNA, and splice-site switching oligomers (SSO) have vast chemotherapeutic potential. Nanoparticulate biomaterials hold promise for DNA and RNA delivery when a means for binding is identified that retains structure–function and provides stabilization by the nanoparticles. In order to provide these benefits of binding, we combined DNA and RNA with protamine—demonstrating association to gold microparticles by electrophoretic, gel shot, fluorescence, and dynamic laser light spectroscopy (DLLS). A pivotal finding in these studies is that the Au–protamine–DNA conjugates greatly stabilize the DNA; and DNA structure and vaccine activity are maintained even after exposure to physical, chemical, and temperature-accelerated degradation. Specifically, protamine formed nanoparticles when complexed to RNA. These complexes could be detected by gel shift and were probed by high throughput absorbance difference spectroscopy (HTADS). Biological activity of these RNA nanoparticles (RNPs) was demonstrated also by a human tumor cell splice-site switching assay and by siRNA delivery against B-Raf—a key cancer target. Finally, RNA:protamine particles inhibited growth of cultured human tumor cells and bacteria. These data provide new insights into DNA and RNA nanoparticles and prospects for their delivery and chemotherapeutic activity.

1. Introduction

Despite the promise of nanotechnology for the delivery of therapeutic nucleic acids, a major current limitation is the inability to combine therapeutic DNA and RNA with nanomaterials to maintain structure and function after bioprocessing and delivery. DNA vaccines [1], siRNA [2], and splice-site switching oligomers (SSO) [3] may have the ability to modulate gene expression in cells, thereby creating a new class of molecular chemotherapeutics. Given the emergent knowledge that alternative splicing plays an important role in the molecular basis of disease [4], there is an important impetus for SSO delivery, in particular, because nanoparticles delivering RNA may be able to elegantly target and correct the disease at the RNA level.

For binding to nanoparticle supports, the majority of work reported thus far has focused on DNA, not RNA. Typical modes of DNA binding involve (1) alkylthiol- or disulfide-terminated oligonucleotides on a metal nanoparticle surface, (2) covalent binding of oligonucleotides to a pre-activated nanoparticle surface, and (3) absorption of biotinylated oligonucleotides on surfaces coated with avidin or streptavidin [5,6]. Indeed, Seferos et al. [7] recently demonstrated that such oligoDNA–gold conjugates, termed "nanoflares," can specifically detect mRNA. Thiol linkage to metal nanoparticles takes advantage of the strong coordination chemistry that exists between some metals and sulfur. Importantly, DNA–Au nanoparticles, functionalized with probe strands terminated in a triethylthiol group, were stable at higher temperatures and in the presence of reducing agents when compared to mono- or cyclic-dithiane species [5]. Furthermore, DNA–Au nanoparticles from the trithiol linker had higher DNA-probe surface coverages than those from mono-thiol or cyclic-dithiane species [6]. However, it is unclear currently whether or not these strategies can be applied to macromolecular DNA vaccine, siRNA, or SSO RNA analogs.

For DNA vaccines, one approach we have patented involves attaching DNA to the surface of gold microparticles via protamine for delivery by gene gun [8]. To enhance stability, several groups have reported that the attachment of proteins to gold nanoparticles

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affords the proteins resistance to temperature and protease degradation [9,10]. In comparison to proteins, nucleic acids are considerably more susceptible to chemical and enzymatic degradation. Recently, we reported that protamine mediates attachment of DNA to gold microparticles and protects the DNA from nuclease degradation [11]. Herein our aim was to investigate whether or not Au–protamine–DNA conjugates enhance chemical stabilization of DNA as well; treating them for weeks to months against accelerated physical, chemical, and temperature degradation; and then testing their vaccine activity in mice.

In addition to its stabilization enhancing properties, protamine is well known for transporting macromolecules into cells. More importantly for this work, protamine is known to condense DNA into nano-structures [12,13] and to deliver antisense oligonucleotides as nanoparticles [14]. Protamine is known, additionally, to exhibit cell penetrating activity [15,16] and is an important component of several cancer targeting systems [17,18]. However, protamine binding to siRNA or SSO RNA, and thus the formation of RNA:protamine nanoparticles and their delivery, has not been described to the best of our knowledge. Finally, we investigated the potential chemotherapeutic utility of these RNA and DNA nanoparticles and their effects on cultured human tumor cell and microbial cell growth.

2. Materials and methods

2.1. Reagents and bio-molecules

Microparticle gold was obtained from DeGussa or from Bio-Rad. Protamine (clupeine) and all other reagents were obtained from Sigma–Aldrich. Plasmid DNA was obtained as previously reported [19]. Oligomer sequences (705 SSO) and siRNA (see below) were a generous gift from Professor Rudy Juliano, University of North Carolina-Chapel Hill, Chapel Hill, NC.

2.2. Nano-conjugation

Nanoparticles were prepared on a Cole-Parmer dual head Masterflex C/L (model #77120-62) pump at a constant rate with microfluidic inner diameter tygon tubing. Gold particles were included in some batches (3–35 mg batch sizes) mixed manually on a vortex at approximately 1 ml/s by first adding protamine (0.01–2 mg/ml) followed sequentially by addition of DNA or RNA (0.01–0.1 mg/ml). Thereafter, the particles were precipitated from 70% ethanol or stored in a 70% ethanol solution until use. In some cases, nanoparticles were separated by high speed density gradient centrifugation coupled to light scatter detection (University of North Carolina-Chapel Hill, Biophysical Instrumentation Core). Alternatively, nanoparticles were confirmed by dynamic laser light scatter spectroscopy (DLLS) in the laboratory of Professor Leaf Huang (University of North Carolina-Chapel Hill, Department of Molecular Therapeutics) or on a Malvern Zetasizer Nano-ZS90 DLLS from a suspension of water or phosphate buffered saline.

2.3. Nano-characterization

UV/Vis and fluorescence spectroscopy of the nanoparticles and nucleic acids were conducted on Thermo Scientific NanoDrop™ instruments, either a NanoDrop 1000 Spectrophotometer or a NanoDrop 3300 Fluorospectrometer.

2.4. Stabilization

Open circle (OC) DNA standard was generated by treating supercoiled DNA with citrate/DMED [20]. Linear (Lin) DNA standard was generated by restriction digestion with XMa1 following the vendor's recommended protocol. Both OC and Lin nucleic acid species were precipitated from ethanol (Spectrum UV grade) (70% alcohol/water), re-dissolved in 1× Tris/NaCl/EDTA (TNE buffer), and stored frozen until use. Anion-exchange HPLC chromatography was performed as described by Molloy et al. [21], except with the employment of a Toso-Haas column and a sodium chloride/aqueous gradient on a Varian Prostar system with DAD detector. Oxidation was accomplished in 0.1% H_2O_2. Two week and one, two, or three month accelerated degradation treatments were conducted in standard cell culture incubators at 5% CO_2 in the presence of desiccant at room temperature or 40 °C or, in some cases, 60 °C. Gamma irradiation treatment was executed by standard protocol (Sterigenics, Inc., NC). For autoclave treatments, particles enclosed in sealed Eppendorf microfuge tubes were exposed to 30-min sterilization cycles "dry." Particles (2–3 mg) exposed to accelerated degradation were then eluted in 1× TNE containing 0.1% SDS for 30 min at 37 °C. Ten microliter aliquots were loaded in 1× load buffer (blue juice, Gibco-BRL) and electrophoresed through 1% agarose gels containing 0.5 μg/ml ethidium bromide for 45 min at 107 V and 440 mA in a Horizon Life technologies gel system running in 1× TBE (Gibco-BRL), and the gels were visualized on a gel documentation station (Syngene, Genetools).

2.5. HeLa 705 splice-site shift assay, siRNA and DNA vaccine biological activity

HeLa 705 cells [22] were seeded into 96-well plates (5000 cells/well) and allowed to attach overnight. Particles (100 μL), containing 0.1–1 mg/ml protamine and 150 nM of either 705 sequence [3,22] or anti-B-Raf siRNA sequence (5′-AUGAUCCAGAUCCAAUUCUdTdT-3′), were added to the well in a total volume of 200–300 μL Opti-Mem (Gibco-BRL). The medium was removed 6–8 h later, 300 μL 10% FBS/DMEM (without indicator) was added, and the cells were allowed to grow for an additional 48 h.

The medium was then aspirated, the cells washed twice with PBS buffer and then cell lysis buffer, luciferase kit reagents were added, and luminescence assayed (ProMega, Madison, WI). For B-Raf, A375 cultured melanoma cells were treated with anti-B-Raf siRNA sequence and B-Raf assayed by western blot according to our previously published procedure for MDR [23]. DNA vaccine studies were conducted as previously described [19,25]. Briefly, HBV plasmid (pdp-SC18) was formulated onto gold particles with a 2 μg pDNA/mg gold target load. Mice were primed with a single dose of DNA vaccine by gene gun onto the shaved skin on the abdomen following PowderJect IACUC protocol under the direction of Dr. Lendon Payne. Two weeks later, blood was collected for hepatitis surface and core antigen ELISA. In some cases, Au–protamine–DNA or Au–spermidine–DNA controls were incubated at 4, 25, 40, or 60 °C prior to gene gun administration. Student's *t*-test was used for statistical analysis of the data; statistically significant differences were assumed at $p < 0.05$.

2.6. Microbiology, cell growth and morphology assays

From a slightly turbid culture of *Escherichia coli* DH10B grown for approximately 6 h at 30 °C, a well-mixed 2 μL aliquot was transferred into a sterile glass tube containing 60 μL of nanosuspension formed just previously by the 1:1 vol/vol addition of protamine (5 mg/ml) to tRNA (1 mg/ml) or M9 media alone controls. The suspension of nanoparticles and *E. coli* cells or controls without nanoparticles were then brought up to 3 ml total volume with M9 minimal medium, mixed, and either 2 or 20 μL inocula volumes were placed onto sterile LB plates and grown overnight. Suspension cultures were grown for 5, 30, 60, 120, or 240 min in the presence of nanoparticles normalized to 10, 3, 1, or 0.3 mg/ml dosages or parallel controls of protamine, tRNA, or BSA. LB plates were then inoculated, grown overnight, and the colonies counted. A growth culture was prepared, 200 μL was spread onto an LB plate, and the plate was allowed to dry in a sterile air flow hood for 10–15 min. A serial dilution series of tRNA:protamine nanoparticles, tRNA (1 mg/ml), protamine (5 mg/ml), or BSA (5 mg/ml) controls were spotted in 3 μL spots onto the pre-spread and dried LB plate and allowed to dry for an additional 15–20 min; subsequently, the cultures were allowed to incubate overnight. Live/dead cell assay was performed per manufacturer's instructions (Invitrogen Corp.) with Nikon TE2000-E optics coupled to a Bio-Rad Radiance 2100 MP Confocal Laser. HeLa 705 cells were plated onto a 96-well plate in DMEM with 5% FBS and grown to 80% confluency. These cells were treated with tRNA (0.1 mg/ml), protamine (1 mg/ml), and the tRNA/protamine complexes (1:1 v/v). The cells were incubated for 6 h at 37 °C. After 6 h, the medium was removed from the wells. 100 μl of PBS were added to the wells with 100 μl of Trypan Blue. The cells were mixed and stained for 5–15 min. The stain was removed from the cells, and the cells were washed with PBS. Images of the cells were taken using an Olympus 1 ×70 inverted brightfield microscope. In some cases, yeast cells were treated and imaged similarly.

3. Results

3.1. Characterization of DNA binding to gold microparticles

Evidence for DNA attachment to gold is shown in Fig. 1. Particles were prepared with DNA attached to gold [11,19], and the supernatant or particle-associated fraction (eluted from the particles) was run by gel electrophoresis (panel A). In comparison to internal load standards, all five batches appeared to have 25–50 ng of DNA loaded per 0.5 mg of gold eluted. Particles were shot into agar gels by a gene gun in the absence (panel B) or presence (panel C) of ethidium dye and a non-fluorescence or fluorescence pattern photographed, revealing the particulate pattern of stained DNA (panel C). Alternatively, a series of five samples were prepared, and the supernatant (sup) or particle-associated fractions (part) were assayed by UV or fluorescence spectroscopy (panel D), indicating a loading efficiency of greater than 90%. Dynamic laser light scatter (DLLS) showed