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Development of a Novel Assay to Determine Nanoparticle Protection of Complexed dsDNA

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ABSTRACT

Nanotechnology is one of the fastest growing fields in pharmaceutical science. In particular, the field of systematic drug delivery is one of the most promising. The goal of systematic drug delivery is to find alternative forms of cancer treatment. This has become very popular research due to the limited effectiveness of radiation and chemotherapy. The goal of systematic drug delivery is to find alternate means of getting the specific drug to the affected part of the body without the side effects on the other portions of the body it goes through. By using drugs with a specific affinity towards certain body parts, there won't be side effects on the body parts while passing through. In this study, nanoparticels (nanogels) loaded with DNA were assayed under a variety of conditions. One issue of importance is the protection these nanogels might provide to the pharmaceutical industry as it moves through the body to a specific target. DNA is used as a simple model for pharmaceuticals. Nanogel, nanogel 10%, and nanogel 15% were loaded with DNA, incubated with a endonuclease inhibitor, and assayed for their ability to protect the DNA complexed to the nanogel. Fluorescence spectroscopy, using a fluorescent indicator served as a basis for the assay. A loading ratio of 8:1 was found to be most effective and preserved near 90% of the complexed DNA with 1 hour complexation. A longer period of incubation resulted in a great loss of DNA. A 15:1 proved to be most effective to protect at the longer time.

Keywords: *nanotechnology, drug delivery vehicles, nanoparticles, systematic drug delivery.*

INTRODUCTION

Today a major part of most people's lives is the ability to use pharmaceuticals and prescription drugs to overcome an illness or ailment. A challenge is that pharmaceuticals can only do so much for an individual and help a limited number of ailments. Over the past few years, much work has been done in pharmaceutical cancer research, specifically in the area of systematic drug delivery. Systematic drug delivery is of great importance to cancer patients due to the possible ability to distribute a certain drug to a specific part of the body without that drug harming other parts of the body on the way such as in chemotherapy side effects. Many techniques are being developed to try to achieve this goal (Torchillin 2000). Some of the many ways being introduced are passive drug targeting, which involves getting high concentrations of the drug in areas of leaky vasculature. Direct application, involves getting the entire concentration of drug to the area of concern (tumors, etc.). Physical targeting is used when the area of concern has an abnormal pH or temperature. Lastly, magnetic targeting is when the drug is attached to a paramagnetic material under an external magnetic field (Torchilin 2000). These are just a few techniques being experimented with today. Some of the challenges with these techniques are: getting the concentrations of drugs to the affected part without them being lost early, getting the drug to the area in high concentrations, and possible side affects to such high concentrations.

A novel area of research in this field is the use of nanogels. A nanogel has a cross-linked core of polyethylenimine and polyethylene glycol. It's high composition of PEG means that it can take on a large amount of water and act as a gel. The actual structure is somewhat random within the nanogel because of synthesizing techniques. It is best described as a 3-dimensional net that expands with the addition of water and contracts when it binds to oppositely charged objects such as dsDNA (dsDNA in this experiment acts as a model). It is suspected that nanogels may be able to protect pharmaceuticals for systematic drug delivery. Drug targeting is the process of getting a transport system, with a drug attached, injected into the body that will have a specific affinity to a certain affected body part (Vinogradov 2005). This means that the drug won't be released until it has reached affected part. By doing this, high concentrations of drug can be delivered and transported through organs, cells, and other systems of the body without causing harm. Current techniques to assess the protection of nucleic acids involve the inefficient extraction of loaded nucleic acids in nanogels and are consequently inadequate for quickly assessing the protection of loaded nucleic acids. The main focus of this paper was to assay how well different concentrations of nanogels can protect nucleic acids (ex. 1.5 μM dsDNA) from endonuclease activity (Turbo DNAse). This simulates the obstacles that may be seen when being administered to the body. By using fluorescence spectroscopy, the amounts of nucleic acids being protected by the nanogel can be seen by comparing with experimental controls. The

degradation process can be determined to see which nanogels protect the best in particular concentrations.

MATERIALS AND METHODS

The materials used for this research were all provided by the University of Nebraska Medical Center, specifically Dr. Joseph Vetro. In order to better understand what materials were used and why, the methods of research should be reviewed. The experiment can be broken down into 6 basic steps: (1) Preparing of the assay buffer, (2) Preparing the nanogel and dsDNA, (3) Preparing the Nanogel-dsDNA complexes, (4) administering Turbo DNAse to break down uncomplexed dsDNA, (5) allowing the time for complexation with the fluorescent tag to take place, and (6) reading the sample with a spectrofluorometer and assessing the amount of dnDNA protected.

Before starting complexation and testing, the proper amount of Sybr Green (Sybr Green is a fluorescence indicator that binds to all dsDNA in solution protected by nanogel) must be quantified. This is done by adding different concentration of Sybr Green to a free 2.5 µM dsDNA solution and begin testing immediately. The results were:

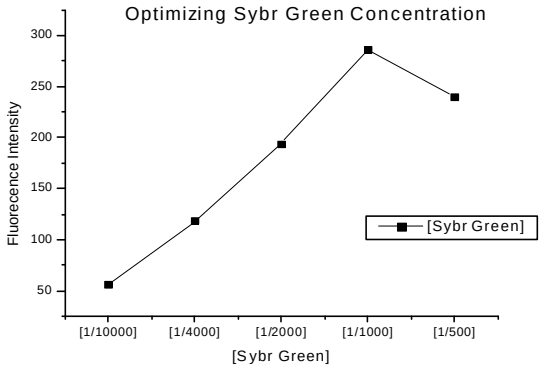

Figure 1.1- Ideal Sybr Green Concentration

The assay buffer is a neutral buffer used for the preparation of both the nanogel and dsDNA and complexation. This was prepared from a standard already developed (Torchillin 2000). The assay buffer consists of 40 mM Tris-HCl, pH 7.4, 10 mM NaCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$. This was made by mixing 3.150 g Trizma- HCl, 0.2922 g NaCl, 0.2856 g $McCl_2$, and 0.0555 g $CaCl_2$ in 475 mL of Deionized H_2O. This showed a pH of 6.77 initially so it was titrated slowly, while mixing, with approximately10 mL of 6 M NaOH to a pH of 7.4. This was diluted further to 498 mL and titrated with NaOH again to maintain the pH of 7.4. The final volume of the solution was 500 mL.

The preparation of the nanogel consisted of weighing out the proper weight of construct (1 mg) and putting it in a 2 mL Eppindorf tube with exactly 2.00 mL of assay buffer, made from step 1, from a volumetric pipette. After equating, this gives a molarity of 5 µM. This solution is sonicated for 5 minutes and then spun for 15 minutes at 4 ºC centrifuge. The solution is then ready to be diluted down to the ratios used in the specific experiment (2:1, 4:1, etc.). The dsDNA is 0.5 mM (6.42 mg/mL) that is diluted in IDT duplex buffer (Vetro). The dsDNA solution was prepared from mixing the 2 strands of dsDNA that had been mixed in duplex buffer according to the weight of each strands (The strands being referred to are the lyophilized 5'-3' and 3'-5').

The nanogel-dsDNA complexes were formed by mixing 2.5 µM dsDNA (9.64 mg/mL, diluted from 0.5 mM stock solution in assay buffer) to form a 2x solution. The nanogel solutions were diluted from the stock (1mg NG=2.3µM primary amines, 6.9 µmoles/mg diluted in assay buffer to form 2:1 nanogel nitrogen to dsDNA primary amines solution) This solution was then diluted to form 4:1, 6:1, 8:1, 10:1, 15:1, and 20:1 ratios. Exactly 1.00 mL of each nanogel solution was added to 2-2 mL eppidorf tubes for each ratio (1 to be treated with Turbo DNase and 1 as an untreated control). Thus giving 12 tubes, each with 1 mL of each ratio in 2 tubes. Then 1 mL of the 2x dsDNA solution was added to all tubes. This gives half the starting concentrations. All samples were then allowed to sit open in room temperature for 1 hour to allow for complexation. 1 tube from each ratio was then treated with 4 units of Turbo DNase (4 U=4 µL), to stop all endonuclease binding activity and ultimately break down all access dsDNA not being protected by the nanogel, and allowed to digest in a 37 ºC bath for 1 hour. This process was repeated with each ratio of nanogel for 5 hours in the bath. Each test was repeated with nanogel 10% (which has 10% less primary binding amines) and nanogel 15% (which has 15% less primary binding amines). All tests were done twice to show reproducibility.

Each tube was then treated with a 1/1000 concentration of Sybr Green and taken to the spectrofluorometer for simple reads against one another. The percent differences between each untreated and treated ratio were plotted to show how well each nanogel construct ratio protected against what it would have done without any Turbo DNase activity.

RESULTS

Our goal was to find the smallest ratio of nanogel to DNA which protects the complexed DNA. The graph below shows the results varied of the study from construct to construct with little to no variation at high N:P ratios for both 1 hour and 5 hour reactions degraded less under endonuclease activity (Turbo DNAse) . The 1 hour complexes protected slightly better than 5 hour, probably due to degradation of

DNA by Turbo DNAse being able to penetrate the nitrogen to phosphorous bonds. This shows that the complex has to be administered shortly after reacting or significant degredation occurs. If significant degredation occurs, vast majorities of complex could be lost before reaching its desired site (of course this reasoning is still hypothetical and not in actually pharmaceutical practice). Almost complete protection was shown from NG 8:1 on up to 20:1. This shows that you can use significantly less NG construct because a 20:1 ratio requires more than twice the NG in initial constructing.

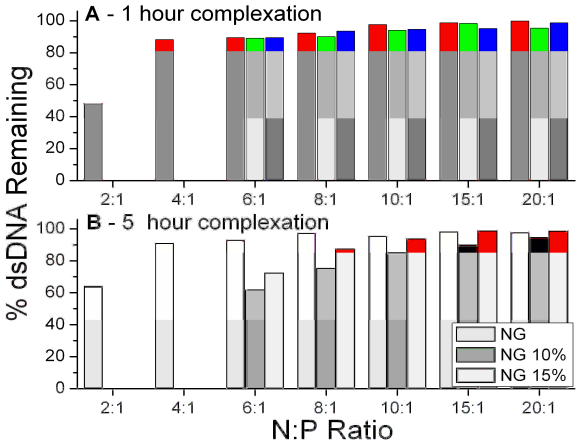

Figure 1.2- 1 hour and 5 hour protection assay

DISCUSSION

The development of this assay was a success. Nanogels are adequate structures for protection of dsDNA when presented with endonuclease activity (Turbo DNAse). The nanogels protect well from N:P rations of 8:1 on up. This shows that anything further than 8:1 NP ratios are not necessary when complexing. This drastically saves nanogel construct used throughout the process. As you can see from the graph above, 1 hour complexation is very adequate and the 5 hour is not needed. Turbo DNAse may penetrate into the constructs in the 5 hour assay, which may explain the lessened protection throughout all N:P ratios. Either way, the 1 hour protection assay proved more valuable in efficiency in the using of nanogel constructs and overall time spent complexing and testing. The assay was a success for the early research in nanotechnology and drug delivery. There are years of research to be done with advancements happening monthly.

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