CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

An increased understanding of normal and disease states allows for a more rational approach to drug design. Defining an objective in the target disease leads to the synthesis of compounds designed to attain that objective. Often this entails the action of the drug at a specific organ in the body, or more specifically, at a definite cellular location, such as the nucleus. This places increased demands on the pharmaceutical scientist to deliver the drug to a specific site, often while minimizing its accumulation in non-target sites. Delivery of the drug to the target site is paramount, as even the most potent drug will be ineffective if it cannot interact with its target.

Advances in molecular biology and chemical synthesis, as well as an increased knowledge of disease states, has led to the ability to design and produce drugs with larger molecular weights. Examples of macromolecular drugs include proteins,[1-3] antisense oligonucleotides,[4,5] and genes[6-8] for gene therapy. These compounds range in size from several thousand to several hundred thousand Daltons. In addition, they must typically reach specific sites

inside cells in order to be active. Their large size and charge limits their access to most compartments, resulting in impaired activity.

Polymeric drug delivery is another area of large molecular weight compounds whose use in pharmaceutics is rising. To overcome one or several problems of a small molecular weight drug, polymeric drug delivery systems are often employed. Some issues which polymeric drug delivery systems are designed to overcome include: problems with solubility, pharmacokinetics, side effects, biocompatibility, stability, immunogenicity, controlled drug release, and cellular distribution.[9-12]

The requirement for the delivery of drugs to specific sites is contrary to the nature of large molecular weight drugs and polymer carriers. The simplest path by which drugs enter cells is diffusion. It has long been accepted that diffusion through a plasma or organelle membrane is restricted to small molecules,[13] which is a major reason why most therapeutics are small molecules. Disadvantages of drugs entering cells by diffusion include a requirement of high permeability and possible efflux (e.g., multidrug resistance due to the efflux of drugs by membrane pumps such as P-glycoprotein[12,14,15]).

The large size and potential charge of these high molecular weight compounds prevents them from entering as many compartments as small molecules.[16] The biodistribution of small molecules is principally governed by their permeability and affinity for biological components with diffusion being their typical method of entry into biological compartments. If the desired balance in solubility and permeability of small drug molecules can be obtained

by chemical modification, the drug should be able to reach most compartments and interact with its target. In contrast to small molecules, large molecular weight material is internalized by endocytosis (discussed in Chapter 2). Adding ligands to macromolecules can target the compound to specific cells and thereby result in increased uptake; but once the material has been endocytosed, it still remains separated from the cell's interior by a biological membrane.

The most common fate of endocytosed material is delivery to the lysosome, where high levels of lysosomal enzymes are present. Drugs sensitive to these enzymes will be quickly degraded if steps are not taken to protect them or to facilitate their escape into the cytosol. The limited number of compartments accessible to macromolecules and probably exposure to a lower pH and degradative enzymes decreases their probability of success. Delivery of these and other large molecular weight compounds to their target site is currently one of the greatest obstacles for their success. Since many drugs today are being designed to work at specific sites within cells, knowledge of the internalization and subcellular fate of macromolecules is paramount when designing a viable therapy. Once the subcellular fate of the macromolecules is well understood, steps can be taken to improve their delivery to the desired cellular compartment.

1.2 Rationale, hypothesis, and research approach

The objective of this dissertation was to further the understanding of the internalization and subcellular fate of macromolecules. The approach was a systematic investigation of a challenging but observable and modifiable drug delivery system for antisense oligonucleotides.

Antisense oligonucleotides are highly specific therapeutics as they are designed to inhibit the cellular production of a single unwanted protein.[17,18] To be active, the antisense oligonucleotide must interact with its target in the cytoplasm or nucleus. This specificity—in addition to the oligonucleotide's large size, potential charge, and stability problems—makes them one of the most challenging macromolecules to deliver. In fact, delivery of antisense oligonucleotides to their target is currently the limiting factor to their success, and the subject of intensive research.[19-21]

The fact that externally administered antisense oligonucleotides can inhibit the synthesis of an unwanted protein is an enigma and is the impetus of this dissertation. Large, highly charged oligonucleotides should not be able to cross the plasma or endosomal / lysosomal membrane before being degraded. They should be internalized by endocytosis and transported to the lysosome where they would be degraded. Despite this expected outcome, the literature contains numerous examples indicating that oligonucleotides escape into the cytoplasm where they actively interact with their target (many examples were reviewed in references [5,22]). These successful interactions led to the formation of the governing hypothesis in this dissertation: despite their

large molecular weight and charge, antisense oligonucleotides delivered to the lysosome can escape into the cytoplasm and actively inhibit the production of the target protein.

Although the hypothesis presumes that the oligonucleotides will be able to escape into the cytoplasm and remain active, the main problem of antisense therapy is the delivery of the oligonucleotide to the cytoplasm and/or the nucleus. One of the primary reasons for the failure of many antisense experiments is poor delivery to the target. The recognition of antisense delivery problems has prompted a surge in research, including this dissertation. It is hoped that the knowledge gained from this work can also be applied to the delivery of other macromolecules, including genes, proteins, and polymer-conjugated drugs.

As a model for the subcellular delivery of antisense oligonucleotides and macromolecules in general, we chose to study the internalization of a neutral water-soluble polymer, which would later be utilized as the vector to deliver the antisense oligonucleotides to the lysosome. The polymers we chose to study were copolymers of N-(2-hydroxypropyl)methacrylamide (HPMA) (reviewed in reference 12). (The rationale and description of the polymer delivery system will be presented in Chapter 2.) Although the HPMA copolymers are almost neutral in charge, their size will allow a comparison to other macromolecules. We will study the kinetics of internalization of the HPMA copolymers including a copolymer with a targeting moiety. Thereafter, we will monitor the internalization and subcellular fate of the HPMA

copolymers by confocal microscopy. With the techniques and information gleaned from these studies, we will then study the fate of the oligonucleotides.

To study the internalization and subcellular fate of oligonucleotides, a polymeric delivery system designed to delivery the oligonucleotides to the lysosome will be synthesized, and characterized. The stability before and after conjugation to the polymer will be determined. The internalization and subcellular fate of the oligonucleotides and oligonucleotide-polymer conjugates will be monitored by confocal microscopy. To determine if the intact oligonucleotide is able to reach its target and inhibit synthesis of the unwanted protein, the activity of the oligonucleotide and oligonucleotide-polymer conjugates will be evaluated.

1.3 Specific aims

1. Determine the fate of HPMA copolymers in cells.
    a. Synthesize and characterize polymers labeled with fluorescent probes, with and without the targeting moiety galactose.
    b. Determine the kinetics of uptake of the galactose-targeted and non-targeted polymers in cells.
    c. Monitor the fate of the polymer in cells by confocal microscopy
2. Determine the fate of oligonucleotides in cells.
    a. Design, synthesize, and characterize a delivery system to deliver antisense oligonucleotides to the lysosome.

b. Determine the effect of polymer conjugation of the oligonucleotide on its stability.
c. Monitor the internalization and subcellular fate of the oligonucleotide and oligonucleotide-conjugates in cells.
d. Measure the activity of the oligonucleotide and its conjugate in cells.

1.4 References

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