CHAPTER 6

SUMMARY AND FUTURE WORK

As discussed in Chapter 2, the use of macromolecules as therapeutic agents and drug carriers has increased dramatically in the past twenty years and there are no indications that this trend will reverse itself. Antisense oligonucleotides are one of the most interesting and promising therapeutic macromolecules. Like many new promising therapeutics, developing potent antisense oligonucleotide therapies has been fraught with many challenges. Despite the problems encountered, interest in antisense oligonucleotides has only increased as previously noted by the increasing number of articles published in this area every month.

Delivery of antisense oligonucleotides, as well as other macromolecules, remains one of the greatest challenges to their success as viable therapeutics. This dissertation explored the internalization and subcellular fate of water-soluble HPMA copolymers, antisense oligonucleotides, and oligonucleotide-HPMA copolymer conjugates. The HPMA copolymers served as more than model compounds for antisense oligonucleotides as their subcellular fate was also of great interest. Despite that HPMA copolymers had been previously studied extensively in vitro and in

vivo and had entered phase I and II clinical trials,[1-3] little was known about their subcellular fate.

The exploration of the mechanisms of action of drugs delivered by HPMA copolymers such as adriamycin[4] will allow for a logical development of subcellular delivery strategies. The first step in such a strategy is to determine the unmodified subcellular fate of the HPMA copolymers.

Except for therapeutics that are active in a wide variety of cell types, organ targeting must be considered before subcellular targeting. Galactose has been found to be one of the most effective targeting moieties for HPMA copolymers in vivo. Conjugating it to HPMA copolymer resulted in rapid uptake of the targeted copolymers in the hepatocytes of the liver.[5] Its small size and neutral charge also made it a good choice for a model targeting moiety. A basic kinetic study of the effect of incorporating galactose on the internalization of HPMA copolymers was preformed. In accordance with previous results, galactose was found to be a potent targeting moiety. A study of the amount of polymer internalized in Hep G2 cells at various polymer concentrations revealed that the amount of nontargeted polymer internalized was consistent with fluid-phase pinocytosis. The amount of galactose-targeted polymer was consistent with receptor-mediated internalization. A time-dependent study and semiquantitative analysis confirmed this result and found that the rate of internalization of the galactose-targeted polymer was two orders of magnitude greater than the nontargeted polymer over a 2-day period.

To determine the subcellular fate of the HPMA copolymers, confocal fluorescent microscopy of fixed and live cells was employed. At short incubation times (typically less than eight hours), the copolymers of HPMA were found to enter cells and remain in small vesicles distributed throughout the cytoplasm. This was consistent with internalization by endocytosis. Polymer targeted with galactose was qualitatively internalized to a greater extent than untargeted polymer. As the incubation time was increased, an increasing proportion of the cells exhibited entry of the polymer into the cytoplasm followed by accumulation in the nucleus. The escape of large macromolecules from endosomes or lysosomes was unexpected. These findings were repeated numerous times with polymers containing other fluorescent dyes including Oregon Green 488, Lissamine rhodamine B, and adriamycin. Fluorescein labeled polymer with and without galactose was also tested. All of the polymers gave similar results although the polymer with adriamycin had only moderate nuclear accumulation. This may have been due to quenching, although additional data is need for any conclusions to be made.

More studies are needed to determine the effects of the fluorescent dyes and drugs on the fate of the polymer. In this dissertation, the dye content was kept to a low level and four different dyes were used to determine if this had any effect on the subcellular fate of the polymers. The only difference between the various polymers was that the nuclear accumulation of the adriamycin labeled polymer was not as extensive as the other polymers

suggesting that quenching or the presence of the fluorescent markers could be altering the polymers fate. Future experiments that might help elucidate the effect of the fluorephores include determining the effect of changing the dye content on the polymers. Subcellular fractionation of radio-labeled polymers (with and without various amounts of fluorescent dyes) could also provide insight into the dyes effects. Subcellular fractionation could also be used to estimate the amount of polymer entering the cytoplasm and nuclei. Extensive controls should be used to insure that the fractionation is working properly and that problems such as transfer of material during cell lysis or contamination of the cytoplasm with endosomes does not occur. Using pH insensitive and photo-stable dyes could also allow semiquantitative analysis of the microscopy.

To confirm that the polymers administered to the cytoplasm would accumulate in the nucleus, various HPMA copolymers were microinjected into the cytoplasm and nucleus of Hep G2 cells. The polymers were found to partition to the nucleus within two hours and remain there for the length of the experiments (up to 4 days). Polymer injected into the nucleus remained there although small amounts of fluorescence were always found in the cytoplasm of all of the microinjected cells. Only minute amounts of polymer was detected in the cytoplasm and less in the nucleus of the polymer-oligonucleotide conjugates PC1 and PC2. The cytoplasmic staining and dark nuclei of the polymer-oligonucleotide conjugates suggest that there are a small number of binding sites with higher affinity in the cytoplasm. This is also consistent with

the persistent low level staining of the cytoplasm seen with the HPMA copolymers without oligonucleotides. More experiments are needed to determine this. Future experiments could include longer incubation times of the polymer-oligonucleotide conjugate PC2. The longest incubation time of the polymer-oligonucleotide conjugate, 24 hours, was a relatively short amount of time for enough polymer to escape from the lysosomes to be observed. Longer incubation times might give more insight into the ability of the polymer to escape form the lysosome after the oligonucleotide was released. It must be kept in mind that the of the oligonucleotide would probably be degraded at long times and hence its fluorescence would not yield usable data. Microinjection of polymer-oligonucleotide conjugates into the cytoplasm could also be used to determine if at higher polymer concentrations, nuclear accumulation was observed.

The subcellular fate of fluorescently labeled oligonucleotides in Hep G2 cells was also studied by confocal microscopy. At very short incubation times (less than two hours) the oligonucleotides remained in small vesicles. At incubation times greater than two hours, fluorescence was found in the cytoplasm and nucleus. The fluorescence of the cytoplasm and nucleus were very similar but there was increased staining of the nucleoli.

In contrast to the cytoplasmic and nuclear staining observed with the polymers, all of the cells were equally stained when incubated with the oligonucleotides. More work is needed to understand the variability of the polymers to escape into the cytoplasm and nucleus. Future experiments

should analyze the cell cycle to determine if it has any effect on the distribution of the polymer.

Attaching the oligonucleotide to the polymer via a non-degradable spacer resulted in retention of all but a miniscule fraction of the fluorescence in small vesicles in the cytoplasm. This indicates that polymer conjugation altered the distribution of the oligonucleotide.

Conjugation of the oligonucleotide to the polymer via the degradable spacer resulted in a distribution of the oligonucleotide that resembled the naked oligonucleotide suggesting that spacer had been cleaved. This also indicates that the cytoplasmic and nuclear fluorescence observed with the free oligonucleotide was not due to degradation as such staining was not observed with the non-degradable spacer, but it returned with attaching the oligonucleotide via the degradable spacer. The separately labeled polymer predominately remained in endosomes / lysosomes. The small amount of polymer that did escape into the cytoplasm tended to remain there in contrast to the nuclear accumulation observed by the HPMA copolymer itself. This evidence supports the hypothesis that oligonucleotide delivered to the lysosomes would be able to escape into the cytoplasm and nucleus.

Another interesting finding was that the Hep G2 cells appeared to actively internalize the oligonucleotide-polymer conjugate in a manner consistent with receptor-mediated endocytosis although no competitive-inhibition studies were preformed.

The findings of this dissertation may not only help in the rational development of subcellular delivery of macromolecules, they also help explain the activity of antisense oligonucleotides. As large charged molecules, it was presumed that oligonucleotides would enter cells by endocytosis and remain in lysosomes until they were degraded. Many studies found that cells incubated with antisense oligonucleotides showed activity suggesting that the oligonucleotide had escaped into the cytoplasm. Using confocal microscopy, we found that antisense oligonucleotides were able to escape from endosomes / lysosomes in relatively short times (~2 hours). Presumably the oligonucleotides were not degraded as similar staining patterns were not observed when the oligonucleotide was attached to the polymer by a non-degradable bond.

Although this work suggests that oligonucleotides, and to a lesser and slower extent, uncharged water-soluble macromolecules such as HPMA copolymer, are able to escape from endosomes and lysosomes, the process is slow and incomplete. Future studies using permeation enhancers such as protein transduction domains[6] could greatly increase the efficiency of cytoplasmic and nuclear delivery although steps would have to be taken to allow for delivery of negatively charged molecules such as oligonucleotides and well as provide for cellular or organ targeting.

The high concentration of antisense oligonucleotide required for antiviral activity, short half-life of the oligonucleotide, and the high fluorescence of vesicles suggest directions for improving antisense therapies. Increasing

the activity and stability of the antisense oligonucleotides, while maintaining high specificity, will provide potent drugs. Better delivery methods are also needed to assure interaction of the antisense oligonucleotide with its intended target.

To better understand the results found in this dissertation and to determine their universality, the subcellular fate of the polymers and oligonucleotides in other cell lines should be examined.

6.1 References

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