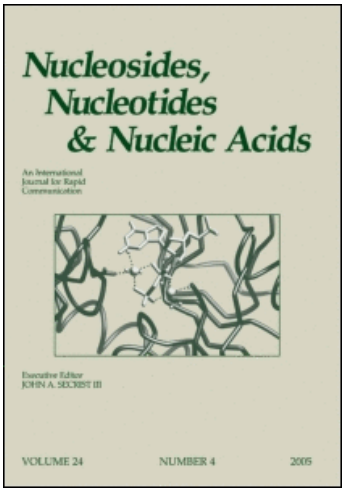

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Specifically Designed Polymeric Nanospheres Increase Cellular Uptake of Unmodified Antisense ODNs

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SPECIFICALLY DESIGNED POLYMERIC NANOSPHERES INCREASE CELLULAR UPTAKE OF UNMODIFIED ANTISENSE ODNs

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ABSTRACT: The cellular uptake and the inhibitory effect of c-*myb* unmodified antisense oligonucleotides reversibly bound to new polymeric nanoparticles in HL-60 cellular system have been found to increase by 50 folds if compared with the free ODN. An initial single dose (320 nM) of the nanoparticle bound unmodified anti*myb* ODN has been able to specifically inhibit HL-60 leukemia cell proliferation for at least 8 days.

Antisense oligonucleotides (ODNs) have been succesfully used to inhibit gene expression both *in vitro* and *in vivo* [1,4]. As the bioavailability of unmodified ODNs is seriously reduced by their rapid degradation in serum and within cells, different chemical modifications were introduced. The phosphorothioate-modified ODNs are nowadays the most widely employed analogues as they were found to be active in several *in vitro* and *in vivo* system. However, contradicting results have been published in the last years, showing sequence-specific mechanisms, whereas others have shown their activity involves a wide range of nonspecific interactions. For example, they interfere with cell growth, cell morphology, viral proliferation, enzyme activities and mRNA expression.

To obtain an efficient delivery of chemically unmodified ODNs through biological membranes, we prepared novel core shell polymeric nanospheres [3], with controlled size and surface nature, specifically designed to directly bind polyanionic antisense agents without requiring other intermediates, that could be toxic upon release inside cells.

These nanospheres contain an inner polymethylmethacrylate core and an outer functional shell consisting of quaternary ammonium groups, able to exchange their bromide counterions with the internucleosidic phosphate groups, so that the ODNs can be directly immobilized to the nanosphere surface through an ion exchange mechanism.

Among the different samples of nanospheres prepared, we selected the nanospheres with narrow size distribution, small medium diameter (460 • 31 nm) and higher surface charge density (177 •mol / g of charged quaternary ammonium groups). These nanospheres allow the adsorption of 0.2 to 12 •mol of unmodified phosphodiester ODNs per gram, whose release is easily obtained in the presence of high NaCl concentration, being minimum in the presence of the culture medium (possible loss of active compound outside cells is prevented). Immobilization of ODNs on the nanospheres proved to be efficient in avoiding nucleases degradation for at least 7 days in the presence of free serum.

For the biological experiments, the following unmodified ODNs, *targeting the c-myb encoded mRNA (codons 2-7) in human leukemia HL60 cells* [4,5], were synthesized and immobilized on the nanospheres:

Sense ODN	**5'-GCC CGA AGA CCC CGG CAC - 3'**
Antisense (AS)	5'-GTG CC**G GGG** TCT TCG GGC - 3'
Reverse ODN	5'-CGG GCT TCT **GGG G**CC GTG - 3'
Scramble ODN	5'-GCT GT**G GGG** CGG CTC CTG - 3'
4 Mismatches ODN	5'-GTA CC**G GGG** TCC TTG AGC - 3'

Delivery of a single dose of AS ODN with the described nanospheres gave rise to a great reduction (50 fold) of the active compound required to obtain the same inhibition of cell proliferation observed with free AS ODN (0.32 •M instead of 16 •M) even after 8 days. To deliver this amount of ODN, the concentration of nanospheres employed didn't give rise to any toxic effects (a 5-fold higher dose of free nanoparticles proved to be devoided of any toxicity as well).

The reduction of cellular proliferation is mainly due to an antisense mechanism as no effect is detectable when any of the control sequences are given immobilized on the nanospheres. This is particularly noteworthy in the case of the control ODNs that maintain the G quartet sequence, as it clearly demonstrates that complexes between unmodified G-rich ODNs and our nanoparticles are able to minimize the possible side

effects due to the well known aspecific interaction of guanosine quadruplex structures with other cellular targets.

Experiments in HL60 cells with radiolabeled and fluorescinated AS ODN immobilized on the nanospheres show an energy dependent faster cellular uptake, which is 50 times higher with respect to the free ODN (9.7 % instead of 0.2 % after 23 hours).

After a single dose of immobilized AS ODN at day 1, the level of *c-Myb* protein was decreased of about 24% at day 2 and about 60% at day 4, suggesting a continuous release of intact ODNs from the nanospheres to the cells, thus simulating a continuous infusion administration.

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