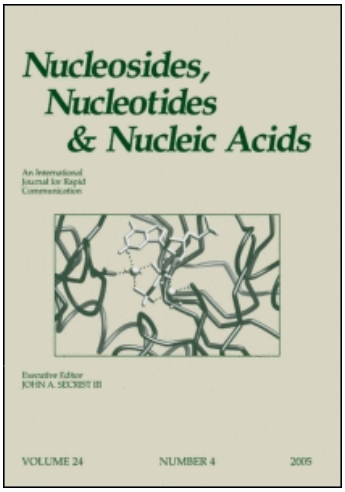

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THE SUPPORT-ON-SUPPORT CONCEPT FOR IN-SITU OLIGONUCLEOTIDE SYNTHESIS ON NANOPARTICLES

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□ *Oligonucleotide-loaded nanoparticles, which are of interest for biomedical application, up to now, could not be prepared by in-situ synthesis, due to difficulty of handling in automated synthesizers. To overcome this problem, we have introduced the "support-on-support" concept. It is based on the reversible anchoring of nanoparticles to the surface of microparticles. These composite beads easily can be used for automated synthesis, being released after completion of chain elongations. As examples, dextran-coated magnetite nanoparticles were attached to polystyrene microparticles through (1) a gelatine or (2) a silica layer. Release involved dissolution of the bonding layer by (1) proteases or (2) alkali.*

Keywords Oligonucleotide; nanoparticles; in situ synthesis; support-on-support concept

INTRODUCTION

Oligonucleotide-loaded nanoparticles have many potential bio-applications,[1] for instance, as probes for labelling and diagnostics, for cell delivery of oligonucleotide therapeutics, for tumour treatment, in array technology, for separation science or for the construction of supramolecular scaffolds and devices. The preparation of oligonucleotide-loaded nanoparticles, up to now, has been done exclusively by post-synthetic immobilization, since nanoparticles cannot be handled in an automated synthesizer. To enable the preparation in an automated synthesis, we have developed a "support-on-support" concept. It includes the following procedures:

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1. The nanoparticles are bound reversibly to microparticles, thus creating a functional "shell" around an unreactive "core." [2,3]
2. Oligonucleotide synthesis is done in a conventional way with these composite beads; the oligonucleotides should be exclusively loaded onto the nanoparticle "shell."
3. After completion of all cycles, the oligonucleotide-loaded nanoparticles are released from the microparticle support.

EXPERIMENTAL

Oligonucleotide syntheses using composite beads were done on an Expedite 8900 DNA synthesizer. ABI crimp-cap style synthesis columns (6 mm length, 6 mm interior diameter) were equipped with 1–2 μm PTFE filters. Due to the flow restriction by these filters, the flow rate was reduced to 50%; delivery times were lengthened accordingly. Otherwise the synthesis cycle was based on the standard 0.2 μmol scale cycle.

The loading of the composite beads was determined by measuring the vis-absorption of detritylation solutions (0.1 M toluene sulfonic acid in acetonitrile). In order to determine the maximum loading of oligonucleotides onto nanoparticles with a non-cleavable bond, test syntheses of $DMTr(dT)_4$ were carried out. If the cleavage of oligonucleotides from nanoparticles after synthesis was desired, the composite supports were first modified with fully protected deoxyguanosine hemisuccinate, by shaking with a 3-molar excess of HBTU, DMAP, and triethylamine in acetonitrile over night. After modification, remaining reactive groups were capped with a mixture of capping reagents A and B in the synthesizer for 30 minutes.

Cleavage of the oligonucleotide protecting groups and release of the oligonucleotide-loaded nanoparticles were done in two separate steps. For deprotection up to 80 mg of the oligonucleotide-loaded core-shell particles were placed into a 2 ml screw-cap microreaction vessel. 1 ml of concentrated (30–32%) ice-cold ammonia was added. The tightly closed tube was then placed in a household microwave oven and irradiated at 450 W for 2 minutes (it is necessary to place a beaker with ca. 150 ml water into the microwave at the same time to avoid popping of the tube content). The microreaction vessels were placed on ice prior to opening; the ammonia was removed by centrifugation, and the core-shell beads were washed once with water.

Olgonucleotides bound to nanoparticles via an ammonia-labile succinate linkage were released during this microwave deprotection step. This treatment does not lead to the release of detectable amounts of nanoparticles.

Cleavage of the magnetite nanoparticles from the "core" was effected by shaking with 0.15 M sodium hydroxide over night. Core particles and some

agglomerated nanoparticles were removed by centrifugation. Suspended nanoparticles were collected by passing the supernatant through a high-gradient magnetic column (Miltenyi) and washing with water.

The quantities of released nanoparticles were determined by UV spectroscopy. For this purpose, aqueous stock suspensions were prepared; the concentration of nanoparticles in these suspensions was determined gravimetrically after evaporation of the water and drying at 55°C for one week. UV-vis spectra of dilutions of the stock suspensions were recorded. This allowed the calculation of calibration curves at wavelengths between 250 and 600 nm. Unknown concentrations of nanoparticles could easily be determined by recording the UV-vis spectra. If the nanoparticles were already loaded with oligonucleotides, readings well outside the absorption wavelengths of the oligonucleotides should be used only.

RESULTS AND DISCUSSION

Suitable "support-on-support" systems must meet stringent criteria: (1) The core microparticle must be non-porous and inert under conditions of oligonucleotide synthesis; (2) the nanoparticle "shell" must be immobilized via reversible bonds to the microparticle "core;" (3) the nanoparticles must contain specific anchor groups for the growing oligonucleotide chains. This anchoring can be ammonia-labile, if analysis of the oligos is desired. For most applications the oligonucleotides will be affixed either by a phosphate ester linkage or by a phosphor-amidate linkage, which are both stable under neutral conditions.

Several routes were explored for realizing the support-on-support concept:

(1) The first approach was to simply test a system composed of silica nanoparticles bound to a polystyrene "core" microparticle.[2,3] Unfortunately, we found, that the silica nanoparticles would not withstand the ammoniacal conditions used for deoxy-oligonucleotide (ODN) deprotection.
(2) Magnetite nanoparticles are known to be inert to alkaline media. Thus, an interference with conventional oligonucleotide synthesis is excluded. In a second approach the polystyrene core particles were prepared in the presence of hydroxyethyl-methacrylate (HEMA), thus producing microparticles with pending poly-HEMA chains. The hydroxyl groups of the latter were activated with carbonyl-diimidazol and coupled via urethane bonds to gelatin-coated magnetite nanoparticles. ODN synthesis on these composites proceeded smoothly, however, the ODN-covered nanoparticles were not released. This may be due either

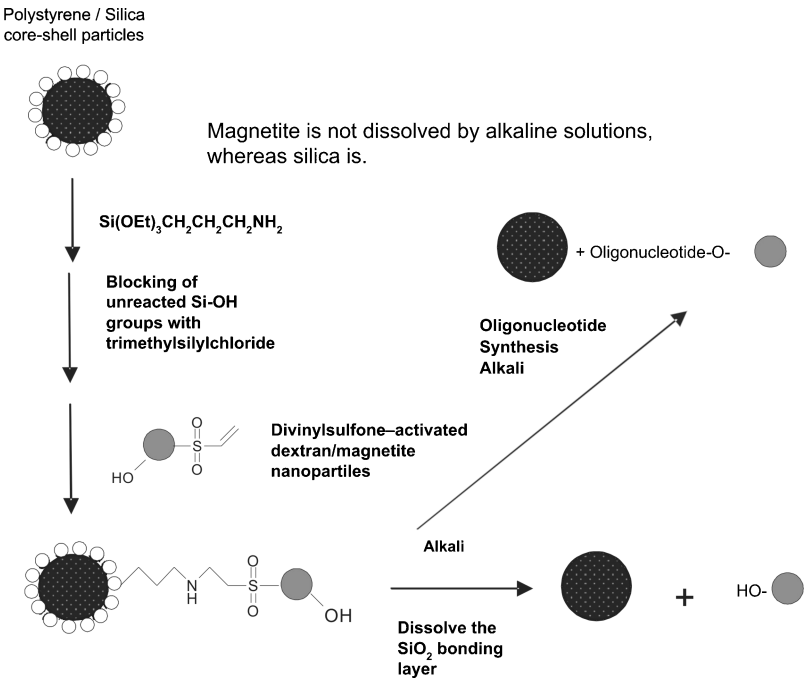

FIGURE 1 Preparation of oligonucleotide-loaded nanoparticles according to the support-on-support concept.

to limited accessibility or entanglement of the nanoparticle "shell" or to kinetic reasons.

(3) The preparation was then reversed by first coating the polystyrene microparticles with gelatin, then attaching the dextran–covered nanoparticles to residual gelatin amino groups via divinyl sulfone. The resulting composite beads gave good results in ODN synthesis. Also, more than 90% of the nanoparticles could be released by enzymatic treatment of the gelatin "bonding layer" with (1). trypsin, (2). protease from Bacillus polymixa.

(4) Excellent results also were obtained, when we simply modified approach no. 1 to using a shell of amino-modified dextran/magnetite nanoparticles instead of silica (Figure 1). ODN synthesis proceeded smoothly on these composite particles, and nearly complete release of the ODN-covered nanoparticles resulted during alkaline deprotection of the oligo. From Table 1 it can be seen, that the capacity of the total composite beads is around 15 μmol ODN/g. Calculating the weight fraction of nanoparticles to ca. 1% and assuming, that only these nanoparticles support ODN synthesis, the loading capacity of the nanoparticles, themselves, should be well beyond 1000 μmol/g.

TABLE 1 Loading capacity of nanoparticles bound to polystyrene microparticle supports

	Core Particles: polystyrene							
	Crosslinked[a]				Highly crosslinked[b]			
Bonding	Gelatin		SiO_2		Gelatin		SiO_2	
Capping[c]	−	+	−	+	−	+	−	+
Capacity in μmol/g	5.2	3.7	15	13.3	3.8	3.7	18.3	15.5
No.	1	2	3	4	5	6	7	8

[a]Prepared with styrene/divinylbenzene = ca. 2:1.
[b]Prepared with styrene/divinylbenzene = ca. 1:2.
[c]Capping: silylation after fixation of nanoparticles.

If the oligonucleotides were attached onto nanoparticles via an ammonia-labile succinate linkage, they could be released into solution by ammonia treatment (see Experimental) without cleavage of the nanoparticles from the microparticle "core." 2 20mers were prepared with average efficiency of 98% (Figure 2).

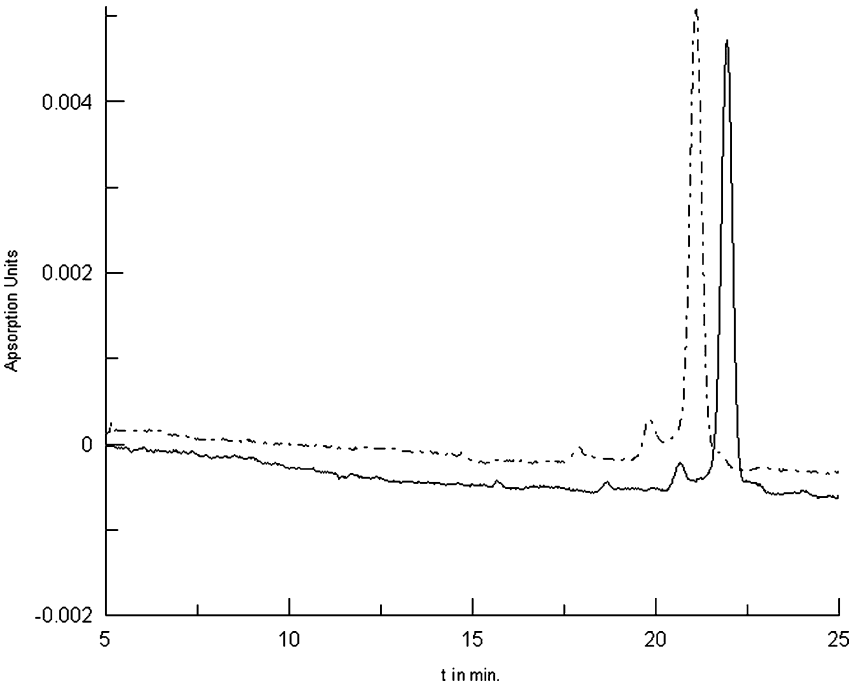

FIGURE 2 Analysis of two samples of the oligonucleotide 5′dCGTTGTAAAACGACGGCCAG3′ prepared on different batches of core-shell beads. Capillary electrophoresis: 100 μm uncoated capillary, filled with a solution containing 7.5% polyethyleneglycol 7500, 1.5% polyvinyl-pyrrolidone in 1× TBE buffer with 3.5 M urea. 10 kV, 20°C.

CONCLUSION

The realization of the "support-on-support" concept leads to a new technology for the synthesis of oligonucleotide-loaded nanoparticles. The key feature is the reversible binding of nanoparticles to a microparticle support,[4] which was achieved using a "bonding" layer, which is dissolved after ODN synthesis. The resulting ODN-covered nanoparticles can be assumed to have an extremely high capacity.

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