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Materials, Nanoscience and Catalysis **AOC**

Synthesis of oligonucleotide-functionalized magnetic nanoparticles and study on their *in vitro* cell uptake†

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Carboxymethyl dextran (CMD) with varying degrees of substitution was prepared and used as biocompatible coating for magnetic iron oxide nanoparticles. An oligonucleotide (19-mer) was coupled to the CMD-coated particles as a model compound for DNA fragments. Transmission electron microscopy investigations on the cellular uptake of the particles by different tumor cell lines demonstrated that both the CMD-coated and the oligonucleotide-coupled particles are internalized by the cells and deposited in cellular endosomes. The nanoparticles prepared have potential applications in tumor diagnosis and therapy. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: magnetic nanoparticles; iron oxides; ferrofluids, oligonucleotides; cell uptake; carboxymethyl dextran; immobilization; transmission electron microscopy; bcr/abl

INTRODUCTION

Magnetic separation techniques have been used in medicine and biotechnology for many years.[1,2] Commonly, polymer-coated magnetic particles with particle sizes up to 5 μm are employed in these techniques. The particles used can interact with cells by simple unspecific adsorption onto the cell surface or, in a more specific way, by reaction of ligands attached to the polymeric shell of the magnetic particles with target molecules on the cell surface. The recent progress in the development of nano-sized magnetic particles offers new perspectives for diagnostic and therapeutic approaches because these nanoparticles are small enough to enter human cells and to interact with components within the cells. The intracellular uptake of magnetic nanoparticles by various cell lines, following the endocytosis pathway, has been described in the literature. In these experiments, iron oxide particles coated with different organic shells including dextran or dextran derivatives,[3–6] albumin,[7] polyethylene glycol[8] or polyethylenimine[9] were used and in some cases specific ligands like a modified HIV-1 tat-peptide[6] or folic acid[8] were immobilized on the polymeric shell. Relatively little is known about the covalent binding of oligonucleotides to magnetic nanoparticles[10,11] and their cellular uptake. Recently, specific oligonucleotide sequences were found to represent marker molecules for tumor diseases.[12] For this reason, oligonucleotide-loaded magnetic particles possess a remarkable potential as new diagnostic or therapeutic tools in tumor treatment, so the cell internalization and the intracellular interaction of such particles with target structures are of considerable interest.

In this paper we report on the preparation of fluid-phase magnetic iron oxide nanoparticles with an optimized carboxymethyl dextran (CMD) coating suitable for the efficient covalent fixation of bioactive molecules. Selected oligonucleotides serving as model structures for specific tumor markers were linked to these nanoparticles and their intracellular uptake into tumor cells was studied.

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EXPERIMENTAL

Materials and methods

All chemicals were reagent grade and used as received unless noted otherwise. A synthetic oligonucleotide with the

sequence 5′-H_2N-$(CH_2)_6$-CCGCTGAAGGGCTTTTGAA-3′ (ON-19) was used. For fluorescence microscopic detection the oligonucleotide was labeled with a fluorescent marker at the 3′-end (Whatman-Biometra). *N*-(3-Dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC, Fluka) was used as coupling agent. Cell culture media and supplements were obtained from Invitrogen.

The determination of the degree of substitution (DS) of the CMD was performed by dissolving a defined amount of polymer in 1 M aqueous NaOH solution and back titration with 1 M HCl against phenolphthalein. The iron(II) and iron(III) contents were determined after dissolution in HCl by conventional titration with $KMnO_4$ and $Na_2S_2O_3$ respectively. The CMD content in the nanoparticle solutions was calculated from the measurement of the absorbance of a CMD–anthrone complex in concentrated sulfuric acid at $\lambda = 625$ nm.[13] Transmission electron microscopy (TEM; CM20 FEG Philips) and photon correlation spectroscopy (PCS; N4 Plus, Beckman Coulter) were used to study the size and morphology of the nanoparticles. Saturation magnetization was obtained from the curve of magnetization recorded with a vibration magnetometer.

CMD

Dextran (Fluka, $M = 15\,000$–$20\,000$; 20.0 g, 0.12 mol) was stirred under nitrogen in a mixture of isopropanol (425 ml) and 14.3 M aqueous NaOH solution (75 ml) for 1 h (Fig. 1). Monochloroacetic acid (29.2 g, 0.31 mol) was added and the suspension was stirred for 90 min at 60 °C. After cooling to room temperature, the isopropanol was removed by decanting and the residue was stirred with methanol (200 ml) for 15 min. The methanol was decanted and the residue was dissolved in water (150 ml), acidified to pH 2 using a Dowex 50 WX8 ion exchanger, and purified by dialysis against water for 36 h. Subsequent lyophilization and drying in vacuum gave CMD (23.7 g) with an average DS of 0.8. Higher DS values, as described in Table 1, were obtained by repeating the given procedure.

CMD-coated iron oxide nanoparticles

Typically, a viscous aqueous dispersion of magnetic nanoparticles was prepared by adding excess ammonia solution (25%) to an aqueous mixture of $FeCl_3 \cdot 6H_2O$ (10 mmol) and $FeCl_2 \cdot 4H_2O$ (6 mmol).[14] The nanoparticle dispersion was separated magnetically and washed repeatedly with water. After addition of water and adjusting the pH with diluted HCl to 1.3, the suspension was warmed to 45 °C and an aqueous solution of CMD (DS = 1.5; 2.21 mmol) was added with stirring. The suspension was stirred for a further 20 min at 45 °C and the coated nanoparticles were separated magnetically. After

Table 1. Effect of the number of carboxymethylations on the overall product yield and the DS value of CMD

No. of carboxymethylations	Overall yield (%)	DS
1	92	0.8
2	84	1.2
3	78	1.6
4	72	2.1

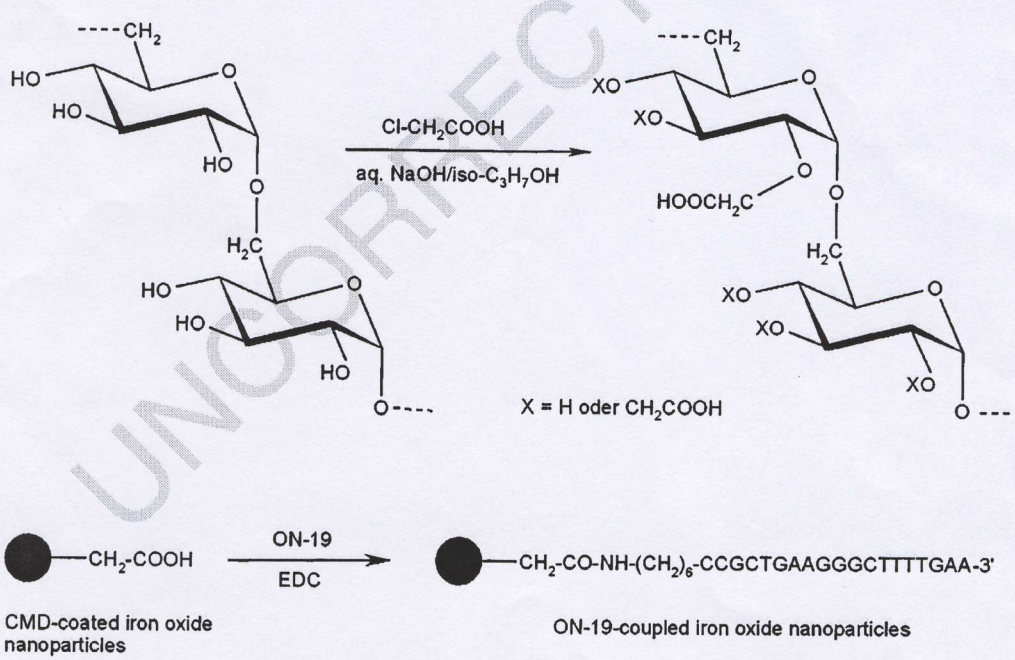

Figure 1. Synthesis of CMD and of ON-19-coupled iron oxide nanoparticles.

Table 2. Characteristics of the stabile CMD-coated iron oxide nanoparticle dispersions

Characteristic	Value
pH (in water)	5.0–5.5
Density (g ml^{-1})	1.07–1.09
Fe(II) content (mg ml^{-1})	9–12
Fe(III) content (mg ml^{-1})	55–70
CMD content (mg ml^{-1})	15–25
Saturation magnetization (mT)	8–11

adjusting the pH with aqueous ammonia to 5–6, the nanoparticles were washed with water until the electrical conductivity of the supernatant was below 500 μS cm^{-1}. Finally, the coated nanoparticles were separated magnetically and homogenized by ultrasonic treatment for 2 min using a Sonoplus UW2200 (Bandelin) device. The characteristics of the iron oxide particle dispersions obtained are given in Table 2.

Oligonucleotide coupling

A solution of EDC (6.0 mg, 0.031 mmol) in phosphate-buffered saline (PBS; 50 μl, pH 5.3) was added to a mixture consisting of an aqueous CMD-coated nanoparticle suspension (500 μl, CMD content: 15 mg ml^{-1}) and 500 μl PBS•. After shaking for 30 min, 100 μl of a solution of ON-19 in water (oligonucleotide concentration: 0.1 nmol μl^{-1}) was added and the reaction was allowed to proceed for 3 h at room temperature. The supernatant was removed by magnetic separation. Purification was performed by dialysis of the nanoparticle suspension against deionized water for 36 h at 4 °C followed once more by magnetic separation. The aqueous ON-19-coupled nanoparticle suspension (500 μl) obtained was stored at 4 °C.

Cell cultures and TEM investigation on intracellular uptake

The breast carcinoma cell line MCF-7 and the chronic myeloic leukemia (CML) cell line K-562 were obtained from DSMZ (Braunschweig, Germany) or ATCC (Rockville, USA). DMEM and RPMI-1640, each plus 10% fetal calf serum (FCS), were used as the culture medium for MCF-7 and K562 cells respectively.

Cells (2.5×10^6 cells per 500 μl reaction vial) were incubated both with CMD-coated and ON-19-containing magnetic nanoparticles, resp.,• in the presence of PBS• and 2 mmol EDTA at 37 °C for 30–50 min. After magnetic separation with a Super MACS (Miltenyi Biotech), the collected cells were washed with 0.1 M cacodylate buffer and incubated in 4% glutaraldehyde in cacodylate buffer for 30 min at 25 °C. Cells are then post-fixed in 1% OsO_4 for 1 h at 25 °C, washed again with cacodylate buffer, dehydrated in an acetone series and embedded in an Araldit/propylene oxide mixture. Ultrathin sections were examined with a Zeiss 900 transmission electron microscope.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in a TRIO-Thermoblock (Whatman-Biometra) using 2.5 μl of reaction buffer (10-fold), 2.0 μl of dNTP mixture (each 10 μM dATP, dGTP, dCTP und dTTP), 1.0 μl of the oligonucleotide-coupled particle solution, 1.0 μl of the bcr-1 and the bcr-2 primer, 1.0 μl of the template DNA, and 0.2 μl of Taq polymerase (Qiagen) filled up with distilled water to an overall volume of 25 μl; 34 cycles (denaturation: 95 °C; annealing: 65 °C; elongation: 74 °C) were run. The PCR products were detected by gel electrophoresis using a 2% agarose gel with ethidium bromide. The template DNA was a pCRII (Invitrogen) plasmid construct containing a 388 bp region covering the K-562 specific bcr/abl breakpoint.

Results and Discussion

The efficient covalent immobilization of oligonucleotides or other bioactive molecules to magnetic nanoparticles requires a sufficient quantity of suitable functional groups on the particle surface. For this reason, we studied the controlled introduction of carboxymethyl groups into dextran, a well-known biocompatible coating material for nanoparticles.[3] Carboxymethylation was performed in the usual way by treating dextran with monochloroacetic acid in an isopropanol–water–NaOH mixture. After a single carboxymethylation step, an average DS related to the anhydroglucose unit of dextran of 0.8 was obtained. According to the literature,[15] DS values higher than 1.0 were obtained by repeated carboxymethylations (Table 1). Degradation of the polymer chains during the carboxymethylation processes could not be detected.

Iron oxide nanoparticles consisting of superparamagnetic, crystalline Fe_3O_4/γ-Fe_2O_3 (magnetite/maghemite) were prepared by conventional coprecipitation of a mixture of ferrous and ferric chlorides in an ammonia medium.[14] The morphology and size distribution of the particles are shown in Figs. 2 and 3. It can be seen from Fig. 2 that the iron oxide particles have a roughly spherical shape. The size of the iron oxide cores was found to be distributed from 3 to 8 nm with a mean particle size of about 5 nm (Fig. 3).

Coating of the iron oxide particles with prepared CMD was performed directly after their formation and acidification of the prepared ferrofluid. Because, normally, it is difficult to observe the polymer coating on the particle surface by TEM, we used PCS to estimate the hydrodynamic diameters of coated particles. The results of the PCS measurements are presented in Fig. 4. Without an additional treatment, the coated particles had hydrodynamic diameters between 250 and 350 nm. After homogenization of the particle solution formed by ultrasonic treatment the hydrodynamic diameters ranged between 150 and 200 nm. Further characteristics of the nanoparticle dispersions prepared are summarized in Table 2.

For our studies of the covalent fixation of DNA fragments onto the surface of the CMD-coated nanoparticles and the optimization of the coupling conditions, an oligonucleotide with the sequence 5′-CCGCTGAAGGGCTTTTGAA-3′ was

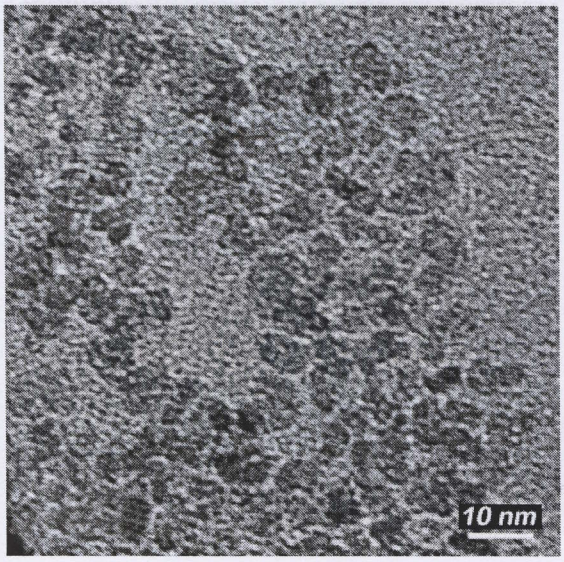

Figure 2. Electron micrograph of iron oxide nanoparticles.

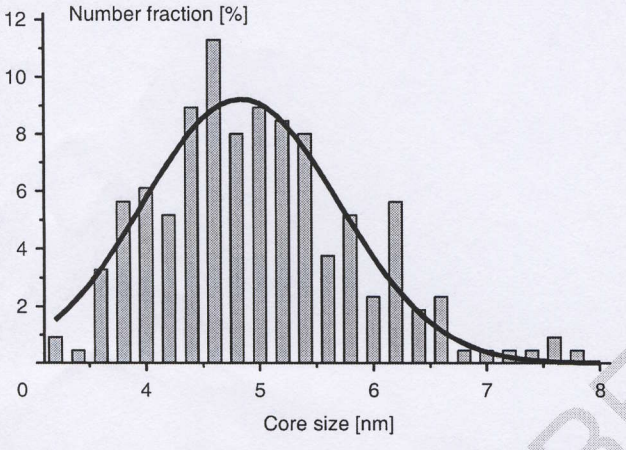

Figure 3. Iron oxide core size histogram of the nanoparticles prepared.

used as a model compound. The 19-mer is a complementary structural fragment of the m-RNA produced from the chromosomal DNA of the so-called BCR/ABL• fusion gene.[16] This genetically abnormal gene is associated with chronic myelogenous leukemia and has considerable clinical relevance for the diagnosis and therapy of this tumor disease.[12] For coupling experiments, an oligonucleotide with an amino linker group at the 5′-end (ON-19) was used and the coupling was performed in PBS at pH 5.3 with a water-soluble carbodiimide (EDC) as coupling agent. The oligonucleotide-containing nanoparticles were purified by magnetic separation and dialysis against water at 4 °C.

A PCR was carried out to detect the immobilized oligonucleotide on the particles and to examine its ability

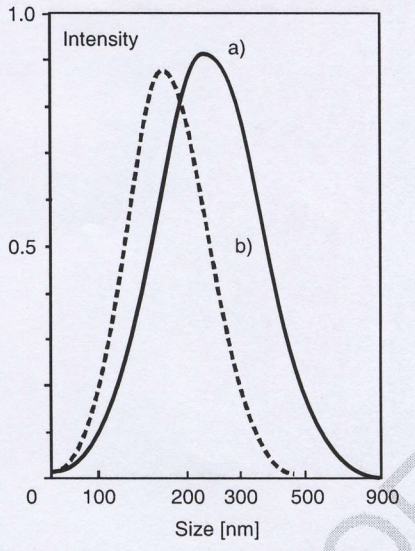

Figure 4. Distribution of hydrodynamic diameters of CMD-coated particles (a) before and (b) after ultrasonic treatment.

to bind to the complementary structure. In a conventional PCR two primers (short, sequence-specific DNA fragments) are necessary to start the amplification of the target structure and the formation of the PCR product. If one primer does not bind to its target region, then no PCR product is detectable. In the PCR reaction performed, the 19-mer oligonucleotide coupled to the nanoparticles acts as the primer binding to the abl region of the bcr/abl gene. A second type of primer was needed to bind to the bcr region (bcr primer). We used two different bcr primers, bcr-1 and bcr-2, binding at different positions of the bcr region. Therefore, two PCR products with different sequence lengths of 305 and 261 bp were expected. The target structure which should be amplified was a representative section from the fusion region of the bcr/abl gene of the K-562 cell line.[16] The PCR product was analyzed by ethidium bromide gel electrophoresis. As shown in Fig. 5, both products could be detected, indicating the successful coupling of functional ON-19 to the magnetic nanoparticles.

In the next step, the uptake of CMD-coated nanoparticles with and without an oligonucleotide ligand by tumor cells was studied. The breast carcinoma cell line MCF-7 and the CML cell line K-562 were used in this study. The cells were cultivated in conventional cell-culture media, incubated with defined aliquots of nanoparticles and separated magnetically. Both tumor cell lines showed a strong interaction with magnetic particles, as indicated by the high number of cells retained within the magnetic field (positive fraction) during magnetic separation. No alteration in growth behaviour could be detected in these cells, even after repeated separation and recultivation, confirming the good cytocompatibility of the CMD-coated nanoparticles.

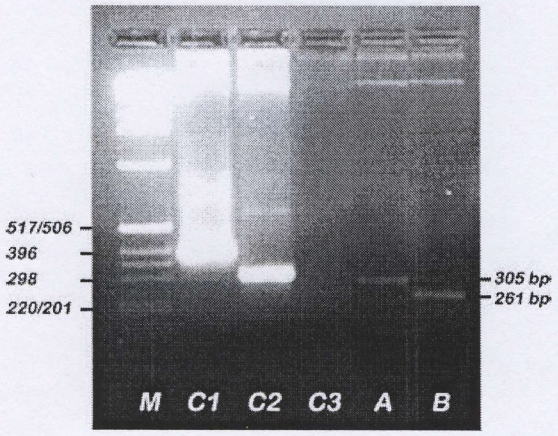

Figure 5. Gel electrophoresis of ON-19-coupled nanoparticles (lane M: 1 kb marker; lane C1: control 1 (bcr-1 primer and abl-1 primer); lane C2: control 2 (bcr-2 primer and abl-2 primer); lane C3: control (bcr-1 primer and CMD-coated nanoparticles); lane A: bcr-1 primer and ON-19-loaded nanoparticles; lane B: bcr-2 primer and ON-19-loaded nanoparticles).

After magnetic separation, the cells obtained from the positive fraction were investigated by TEM. Figs 6 and 7 show transmission electron micrographs of MCF-7 cells treated with CMD-coated and ON-19-coupled nanoparticles respectively. The phenotype of the particle-loaded cells appears normal. It can be observed that both types of nanoparticle form particle agglomerations of up to several micrometers in diameter inside the cytoplasm of the cells. Some of the agglomerations are located close to the nuclear membrane of the cell. Obviously, these particle agglomerations are surrounded by a vesicle membrane. Similar results have been found using the K-562 cell line.

In addition, MCF-7 cells were incubated with CMD-coated nanoparticles bearing a fluorescence-labeled oligonucleotide. Fluorescence microscopy of these cells confirmed the presence of nanoparticles within the cytoplasma•.

It can be assumed from these results that both the CMD-coated and the ON-19-coupled nanoparticles are internalized into the cells by the conventional fluid-phase endocytosis pathway and that they are located in cellular endosomes within the cytoplasm.

CONCLUSIONS

CMD represents a readily accessible, highly cytocompatible coating material for iron-oxide-based nanoparticles. Not only is it able to stabilize the nanoparticle colloid by its carboxymethyl groups, but it can also provide suitable anchor groups for the covalent fixation of biomolecules. The anchor groups content per monomeric repeating unit of the polymer can be properly controlled during the synthesis. In this study, an oligonucleotide with 19 bases and an amino linker at the 5′-end was coupled to the carboxyl groups of CMD-coated nanoparticles via amide formation using a simple coupling protocol.

The intracellular uptake of functionalized magnetic nanoparticles is an important precondition for a specific interaction between ligands coupled to the nanoparticles and intracellular receptors. TEM investigations on the uptake behaviour of the functionalized particles by these different tumor cell lines have demonstrated that both the CMD-coated and the ON-19-coupled particles are internalized by the cells and deposited in membrane-surrounded cellular endosomes. Further studies will now be undertaken to examine the behavior of internalized particles within the cells, especially

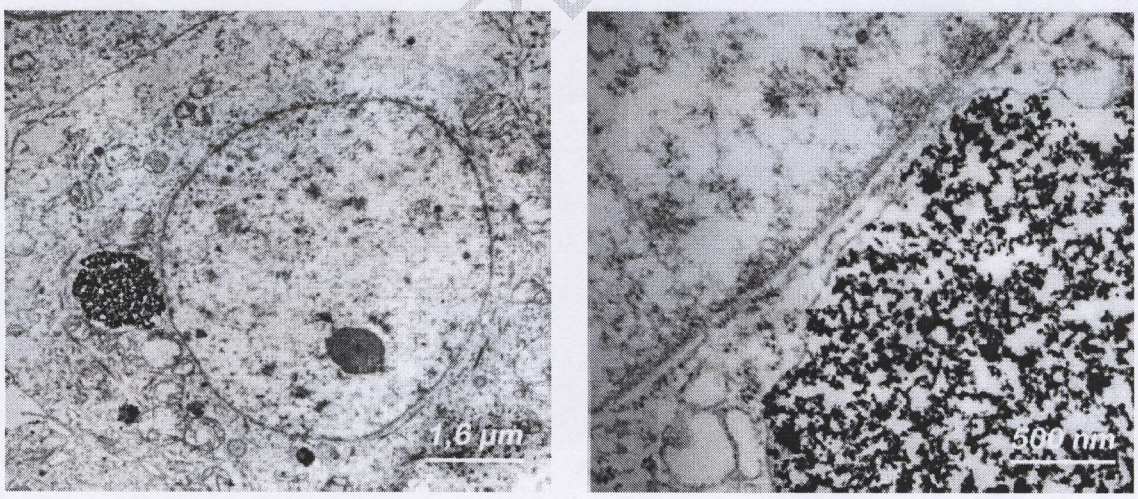

Figure 6. Transmission electron micrographs with different magnification of MCF-7 cells after incubation with CMD-coated nanoparticles.

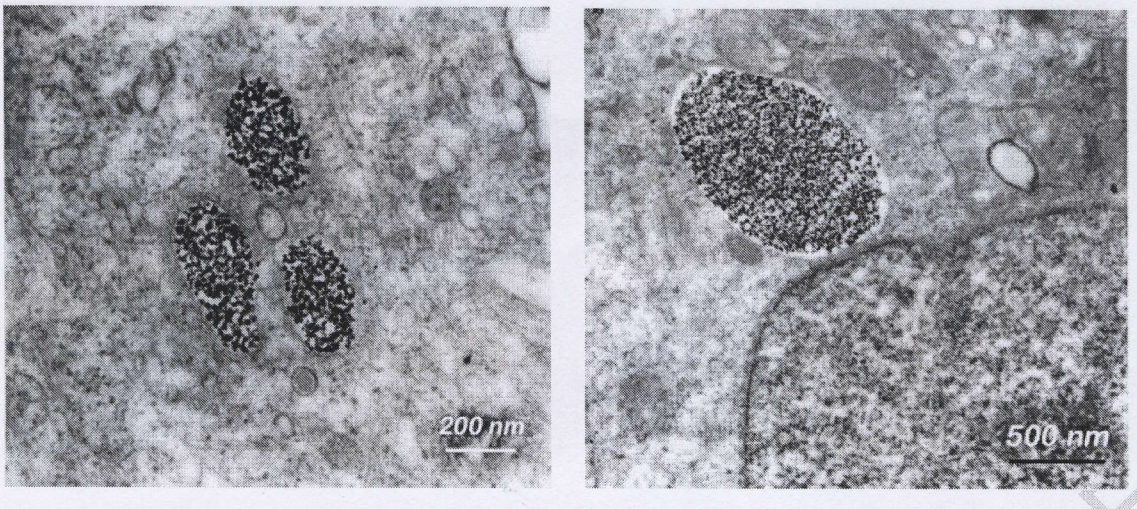

Figure 7. Transmission electron micrographs of MCF-7 cells after incubation with oligonucleotide-coupled nanoparticles.

their release from the endosomal vacuoles and interaction with intracellular target structures.

Acknowledgements

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Queries from the Copyeditor:

AQ1 "puffer". Presumed you mean "buffer"? Hence, superfluous here because of P*B*S, and so deleted.

AQ2 "resp." With respect to what? To use respectively there must be an explicit 1 : 1 correspondence between items (i.e. x and $y = 1$ and 2 resp. implies $x = 1$ and $y = 2$. No other (English) interpretation allowed. Do you mean "were incubated either with CMD-coated or with ON-19 ... nanoparticles in the pres..."? "and" indicates the cells were in contact with both in the same solution. Perhaps "resp." is just superfluous here if you mean "and".

AQ3 "puffer". Presumed you mean "buffer"? Hence, superfluous here because of P*B*S, and so deleted.

AQ4 BCR/ABL here. Why not bcr/abl as elsewhere?

AQ5 "cytoplasma"? "cytoplasm"?