Magnetic Nanoparticles

DOI: 10.1002/anie.200704735

dsRNA-Functionalized Multifunctional γ-Fe_2O_3 Nanocrystals: A Tool for Targeting Cell Surface Receptors**

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The innate immune response is the first line of defense against infectious diseases. The discovery of the family of Toll-like receptors (TLRs) in species as diverse as the fly *Drosophila melanogaster* and also in mammals and humans, as well as the recognition of their role in distinguishing molecular patterns that are common to microorganisms and viruses, have led to a renewed appreciation of the innate immune system.[1] In mammals there are at least ten different (known) TLRs, which are highly phylogenetically conserved and recognize specific components conserved among microorganisms and viruses. Activation of inflammatory responses by TLRs may require the assembly of receptor signaling complexes, including other transmembrane proteins which influence signal transduction. The TLR-induced inflammatory response is dependent on a common signaling pathway that is mediated by the adaptor proteins. The interaction of the different TLRs with distinct combinations of adaptor molecules creates a platform to which additional kinases, transacting factors, and other molecules are recruited. These events lead, in a final step, to gene regulation.[2] The Toll-like receptor 3 (TLR3) specifically recognizes double-stranded RNA (dsRNA), which is a by-product of viral replication and serves as the signature molecule for viral infection.[3] Activation of TLR3 by dsRNA—poly(I:C) (polyinosinic-polycytidyl acid)—induces the activation of the transcription factor NF-kB (nuclear factor-kappa B) as well as of interferon-α/β (IFN-α/β).[4]

One of the exciting new areas of research is the application of magnetic nanoparticles to biological systems, including for targeted drug delivery, magnetic resonance imaging (MRI), biosensors, rapid biological separation, and hyperthermic treatment of tumors.[5] Nanoparticles are attractive probe candidates because of their 1) small size (1–50 nm) and correspondingly large surface-to-volume ratio, 2) chemically tailorable physical properties which directly relate to size, composition, and shape, 3) unusual target-binding properties, and 4) structural robustness. The size of a nanomaterial can offer an advantage over common solids, because a target binding event involving the nanomaterial can have a significant effect on its physical and chemical properties, thereby providing a mode of signal transduction not necessarily available with a bulk structure made of the same material.

The understanding of the interaction between nanostructured materials and living systems is of fundamental and practical interest, and it opens new doors to a novel interdisciplinary research field. Recent reports indicate that magnetic nanoparticles, such as Fe_3O_4, conjugated with various targeting molecules or antibodies, can be used to target specific cells in vitro.[6] However, the noncovalent modification of the surface of nanoparticles has a serious limitation for biological applications, because the exposed metal ion on the surface of the nanoparticles may be cytotoxic, as shown in in vivo models.[7] To overcome this drawback our research has focused on the development of suitable biocompatible materials for the surface coating and functionalization of nanoparticles by using multifunctional polymers that simultaneously bind to inorganic nanoparticles and target molecules through specific anchor groups. Furthermore, they carry a detector molecule that allows the fate of the nanoparticle to be monitored by optical methods. Most striking is the observation that only a small fraction of intravenously administered monoclonal antibodies reach their parenchymal targets in vivo.[8] By virtue of their magnetic properties, iron oxide nanoparticles can not only be used for efficient drug targeting but also for detecting tumors by magnetic resonance imaging (MRI).[9]

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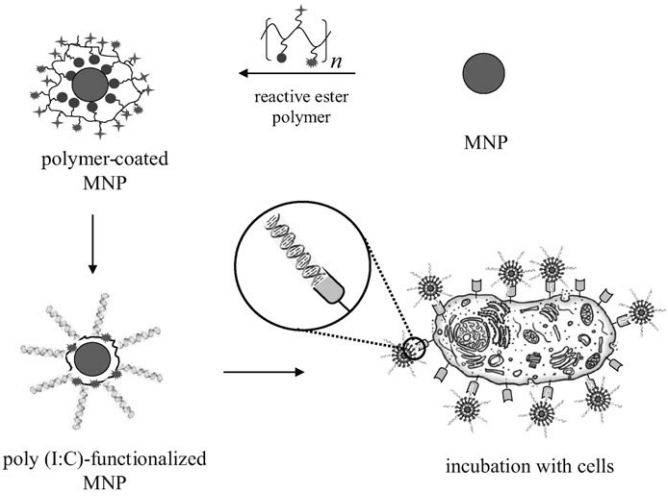
[**] We are grateful to the German Science Foundation (DFG) and the Materials Science Center (MWFZ) of the University of Mainz for partial support.

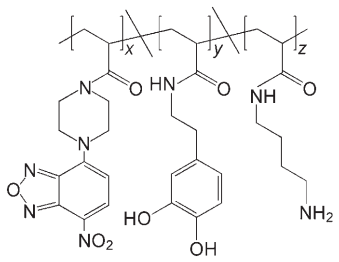
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

Herein we introduce a novel multifunctional polymeric ligand for the immobilization of dsRNA poly(I:C) on γ-Fe_2O_3 nanoparticles that negates the multistep functionalization of nanoparticles. The ligand has the following features: 1) a dopamine-based anchor group which is capable of binding to many metal oxides (for example, Fe_2O_3, TiO_2),[10] 2) a fluorophore (as an optical marker), and 3) a reactive functional group which allows the binding of various biomolecules onto inorganic nanoparticles. Poly(I:C) contains a phosphate group at the 5′ end which makes it amenable to form phosphoramidates through 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) coupling reactions.[11] The biological activity of these poly(I:C)-functionalized magnetic nanoparticles was demonstrated on kidney cancer cells Caki-1 (human renal cell line). The specific binding of the nanoparticle–poly(I:C) complex to the cell receptors is illustrated in Scheme 1.



Scheme 1. Modification of a magnetic nanoparticle (MNP) with a multifunctional polymeric ligand and linkage to dsRNA. The polymer carries a fluorescent dye and amine moieties, which allow conjugation to poly(I:C) through formation of a phosphoramidate by EDC coupling. The poly(I:C)-functionalized nanoparticles were incubated with Caki-1 cells, which bear TLR3 receptors on their surface for specific binding.

Ferrimagnetic γ-Fe_2O_3 nanoparticles were synthesized by thermal decomposition of iron pentacarbonyl, as reported elsewhere.[12] The experimental details are given in the Supporting Information. The γ-Fe_2O_3 nanoparticles were functionalized using a multidentate functional copolymer (Scheme 2) carrying catecholate groups as surface-binding ligands for the iron oxide nanoparticles, a fluorescent dye for optical detection, and free amino groups for further biofunctionalization through the attachment of poly(I:C) ligands. The average size of the particles was estimated using TEM; the apparent size difference between unfunctionalized (Figure 1 a,b) and functionalized particles was not significant, thus suggesting a thin polymer coating (see Figure S1 in the Supporting Information). The Mössbauer spectrum of the unfunctionalized nanoparticles at room temperature is shown in Figure 1 c. As maghemite is superparamagnetic when the



Scheme 2. Multifunctional copolymer containing 3-hydroxytyramine (dopamine) as an anchor group for the binding of metal oxides, piperazinyl-4-chloro-7-nitrobenzofurazane (pipNBD) as a fluorophore, and a free amine group for the conjugation of poly(I:C).

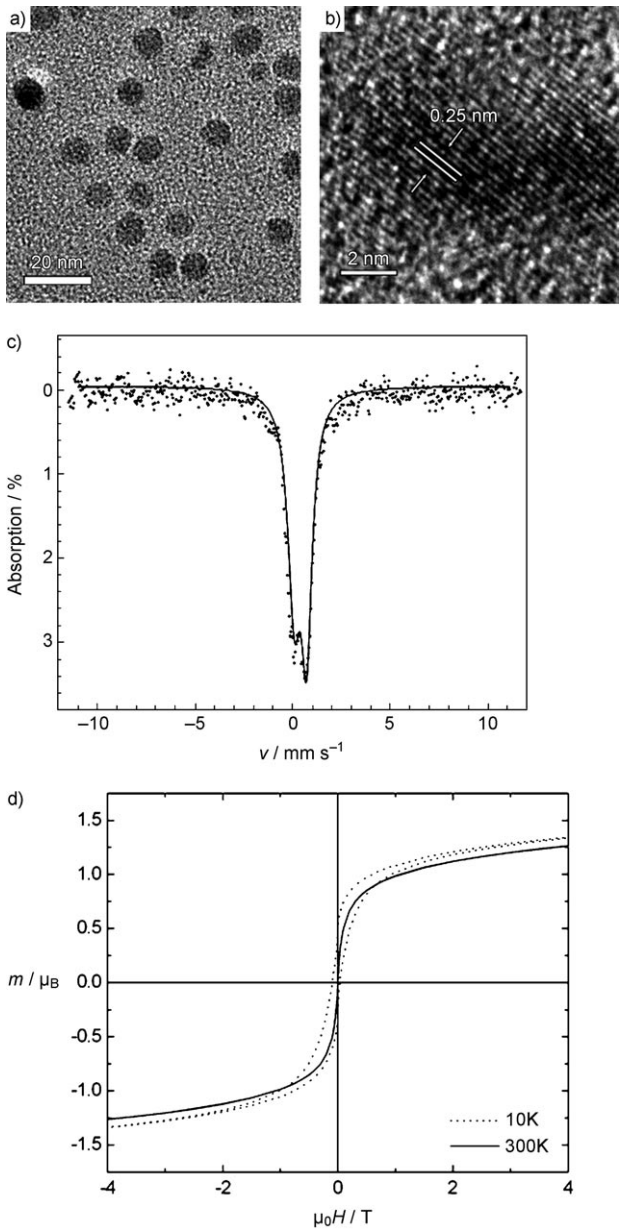


Figure 1. a) TEM images of γ-Fe_2O_3 nanocrystallites. Overview image (a) and high-resolution (HRTEM) image (b) of unfunctionalized particles. c) Mössbauer spectrum of maghemite nanoparticles at room temperature. d) Hysteresis curve for maghemite nanoparticles obtained at 300 and 10 K. m: magnetic moment, $\mu_0 H$: induction field.

particles are smaller than 9 nm, the Mössbauer spectrum contains only a single doublet. The Mössbauer parameters at room temperature ($IS = 0.37(3)\ mm\,s^{-1}$, $QS = -0.35(2)\ mm\,s^{-1}$) are compatible with bulk ferrimagnetic maghemite.[13] The quadrupole splitting deviates slightly from the literature value because of the large fraction of nanoparticles at the surface compared to their volume. The hysteresis curve for the γ-Fe_2O_3 nanocrystallites in Figure 1d indicates superparamagnetic behavior, without any hysteresis at room temperature. The magnetization data indicate a magnetization of 65 $emu\,g^{-1}$ for the γ-Fe_2O_3 nanoparticles at 40 kOe.

The study was carried out with the commercially available dsRNA poly(I:C) at a final concentration of 2 $mg\,mL^{-1}$. Poly(I:C) contains phosphate groups at its 5′ end which makes it susceptible to immobilization as a phosphoramidate on the γ-Fe_2O_3 nanocrystal through EDC coupling.[11,14] The gel electrophoresis in Figure 2 shows in lane 1 pure poly(I:C)

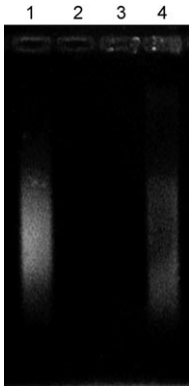


Figure 2. Agarose gel. Lane 1: poly(I:C) (1:10 dilution in MeIm, 0.1 M, pH 7.5 buffer) as positive control. Lane 2: polymer-functionalized γ-Fe_2O_3 nanocrystals. Lane 3: polymer-functionalized γ-Fe_2O_3 coupled to poly(I:C) (EDC coupling). Lane 4 (as a second control): the sample of lane 3 after heating at 75 °C for 5 minutes, thereby breaking the amide bond between the polymer–nanoparticle hybrid and the poly(I:C).

(1:10 dilution in MeIm, 0.1M, pH 7.5 buffer) for comparison, in lane 2 polymer-functionalized maghemite nanoparticles, and in lane 3 poly(I:C)-bound polymer-functionalized maghemite nanoparticles.[14] To verify the bonding between the dsRNA and the functionalized nanoparticles, the complex was heated up to 75 °C for 5 minutes and loaded onto the agarose gel. The presence of a band (lane 4 in Figure 2) confirmed that the poly(I:C) was detached from the polymer-functionalized γ-Fe_2O_3 nanoparticles.

The cells used in this study were Caki-1, which were commercially available and therefore avoided the extensive work of isolating and culturing from fresh tissue. The cells were cultured under the conditions described previously until a confluence of 95–100 % was reached.[15] The expression of TLR3 on the cells was determined by using the (reverse transcriptase)-polymerase chain reaction (RT-PCR) and immunocytochemistry. The RNA was isolated from the cells and the integrity of the isolated RNA was checked by standard gel electrophoresis (1 % agarose). The total RNA was reverse-transcribed into cDNA and was then amplified by PCR. Subsequently, the amplified PCR products were analyzed by gel electrophoresis (2 % agarose) with ethidium bromide staining together with a DNA standard (100 bp DNA ladder; Figure 3a). The 2 % agarose gel showed an

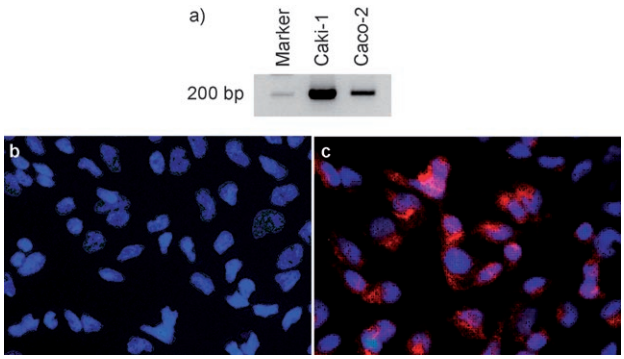


Figure 3. a) Expression of TLR3 mRNA on Caki-1 and Caco-2 cells (208 base pairs). b,c) Immunofluorescence of TLR3 in Caki-1 cells. b) Control experiment with preimmune serum: no signal is observed. c) TLR3 monoclonal antibody and the corresponding Texas Red fluorophore conjugated with secondary antibodies (red). The red fluorescence signal is clearly visualized on the surface of the Caki-1 cells. The cell nuclei were stained blue with 4,6-diamino-2-phenylindole (DAPI).

expected band of approximately 208 base pairs. Caco-2 cells were used as a positive control of hTLR3 mRNA expression to confirm the specificity of the primers used and the PCR, since it has been previously reported to express potentially functional TLR receptors such as TLR3 and TLR9.[16] The RT-PCR results demonstrated that human TLR3 (hTLR3) is expressed with 208 base pairs in Caki-1 cells.

An immunodetection technique was used to visualize TLR3 expression on Caki-1 cells (Figure 3b,c). After a blocking step, the cells were incubated with TLR3 mouse monoclonal antibodies raised against full-length hTLR3 (human origin). Secondary antibodies (goat anti-mouse IgG) conjugated with Texas Red was incubated with the Caki-1 cells and the nuclei were stained with 4,6-diamino-2-phenylindole (DAPI; Figure 3c). Subsequently, the cells were analyzed by optical light microscopy, by using a reflected light fluorescence attachment at emission wavelengths of 456 nm and 620 nm to visualize the DAPI staining and the secondary antibody, respectively. Control experiments on the preimmune serum showed that no signal is derived from the antibody (Figure 3b). However, immunostaining by means of the red fluorescence provided by the conjugation of the secondary antibody to the Texas red fluorophore proves the expression of TLR3 on the Caki-1 cells. This finding is in accord with the RT-PCR results (Figure 3a).

Despite the fact that TLR3 have been described to be internalized in subcellular compartments in some types of cells, such as monocyte-derived immature dendritic cells (iDCs),[17] TLR3 can also be found at the surface of human colonic cancer cell line T84[18] and human lung fibroblast cell line MRC-5 to sense viral infection playing physiological roles in antiviral innate immunity.[17a] However, the cellular local-

ization of such receptors is still poorly understood and under discussion.

An XTT assay (XTT = 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) was performed to assay the cell proliferation of polymer-functionalized γ-Fe_2O_3 nanocrystals. Caki-1 cells were cultured in 96-well plates at 3×10^4 cells per well according to the cell-culture procedure described in Ref. [15]. The cells were incubated with functionalized γ-Fe_2O_3 nanoparticles (at concentrations of 10, 50, and 100 $mg\,mL^{-1}$) in triplicate for 12 h. Cells were washed once with phosphate-buffered saline (PBS) and the cell viability was determined with the XTT assay.[19]

Figure S4 in the Supporting Information shows the effect of the functionalized γ-Fe_2O_3 nanocrystals on the Caki-1 viability. It can be seen that polymer-functionalized nanoparticles had almost no effect on the cell proliferation when compared with the control sample. Concentrations of 10 and 50 $\mu g\,mL^{-1}$ did not harm the cells, while concentrations of 100 $\mu g\,mL^{-1}$ caused 15% cell death. As the toxicity of the polymer-functionalized nanoparticles is very low, even at the higher concentrations, γ-Fe_2O_3 can be used as an efficient vehicle for carrying poly(I:C).

The phase-contrast microscopy images in Figure 4 show the extent to which the poly(I:C)-conjugated γ-Fe_2O_3 nanoparticles are anchored to the cell walls. For this study, Caki-1 cells were incubated with well-dispersed poly(I:C)-coupled

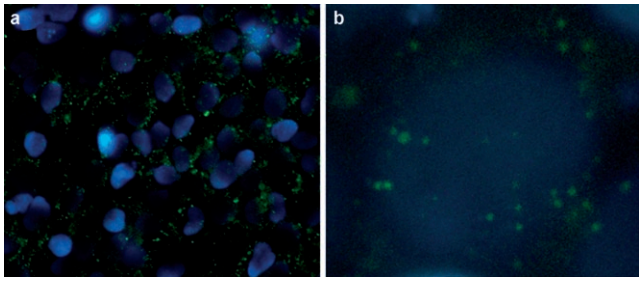


Figure 4. Light microscopy images of Caki-1 cells incubated with poly(I:C)-coupled γ-Fe_2O_3 nanocrystals functionalized with multifunctional polymer carrying a fluorescent dye (green). Poly(I:C)-conjugated nanoparticles bind specifically to the TLR3 expressed on the Caki-1 cells (a). b) Higher magnification image clearly showing the nanoparticles on a single cell. Nuclei were visualized by staining with 4,6-diamino-2-phenylindole (DAPI; blue).

polymer-functionalized maghemite nanoparticles (37 °C, 5% CO_2, 3 h). The cells were then stained with DAPI to visualize the cell nucleus (blue). Subsequently, they were analyzed by fluorescence microscopy at emission wavelengths of 456 nm and 530 nm to visualize the DAPI staining and the fluorophore-coupled maghemite nanoparticles, respectively. The green fluorescence in Figure 4a,b shows the presence of functionalized γ-Fe_2O_3 as carriers of poly(I:C) around the cell walls expressing TLR3. Controls were performed with polymer-functionalized γ-Fe_2O_3 nanocrystals under similar conditions but in the absence of poly(I:C). In this case the nanoparticles were not observed either on the cell surface or inside the cells (see Figure S5 in the Supporting Information). In a further step to confirm the affinity of poly(I:C) as a ligand specific for TLR3, human monocytes—which are reported[20] not to contain TLR3 receptors—were used as negative controls. Mononuclear cells were purified from human peripherical blood by Ficoll–Hypaque gradient centrifugation. Figure S6 in the Supporting Information shows that no nanoparticle attachment was observed after incubation of the dsRNA-functionalized nanoparticles with the human monocytes. The interaction between the nanoparticles and cells depends, in addition to other factors such as size and shape, on the surface aspects of materials that determine the cell behavior. Thus, γ-Fe_2O_3 nanocrystals with a definite size have been prepared that are surface-functionalized with a polymer and coupled to poly(I:C) for targeting cell-expressed surface receptors. In this way highly specific cell receptors can be addressed and endocytosis prevented, since the dsRNA-functionalized nanoparticles show a high affinity for the cell membrane.

This approach opens up new opportunities for the selective marking of cells by using the magnetic properties of the nanoparticles which may be of crucial interest for the development of cellular therapies. Site-specific delivery of drugs and therapeutics can significantly reduce the potential toxicity of a drug and increase its therapeutic effects. A greater understanding of the processes by which TLRs regulate adaptive immunity may yield not only improved ways to treat infectious diseases but also new approaches for the treatment and prevention of allergic and certain autoimmune disorders.

Received: October 12, 2007
Revised: January 21, 2008
Published online: May 15, 2008

Keywords: biosensors · cell recognition · cell surface receptors · maghemite · nanoparticles

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