

# NanoBio-Tools for Selective Activation of Toll-like receptors (TLRs)

M. I. Shukoor<sup>a</sup>, F. Natalio,<sup>b</sup> N. Glube<sup>c</sup>, M. N. Tahir<sup>a</sup>, H. A. Therese<sup>a</sup>, V. Ksenofontov<sup>a</sup>, P. Theato<sup>d</sup>, P. Langguth<sup>c</sup>, J-P Boissel<sup>e</sup>, H. C. Schröder<sup>b</sup>, W. E. G. Müller<sup>b</sup>, and W. Tremel<sup>a\*</sup>

[a] Institut für Anorganische Chemie und Analytische Chemie, Johannes Gutenberg-Universität, Duesbergweg 10-14, D-55099 Mainz (Germany), [tremel@uni-mainz.de](mailto:tremel@uni-mainz.de)

[b] Institut für Physiologische Chemie, Johannes Gutenberg-Universität, Duesbergweg 6, D-55099 Mainz (Germany)

[c] Institut für Pharmazie, Johannes Gutenberg-Universität, Staudingerweg 5, D-55099 Mainz (Germany)

[d] Institut für Organische Chemie der Johannes Gutenberg-Universität, Duesbergweg 14, D-55099 Mainz (Germany)

[e] Pharmakologisches Institut, Johannes Gutenberg-Universität, Obere Zahlbacher Strasse, 55131 Mainz (Germany)

## ABSTRACT

The immobilization of polyinosinic-polycytidylic acid [poly(I:C)] on  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles via the phosphoramidate route using a multifunctional polymer is reported. The dsRNA coupled nanoparticles were used to visualize the Toll-like (TLR3) receptors at the cell surface. The presence of TLR3 was demonstrated independently in the Caki-1 cell line by RT-PCR and immunostaining techniques.

**Keywords:** magnetic nanoparticles, TLR receptors, ds RNA Poly(IC), specific binding, cancer cells.

## 1 INTRODUCTION

One of the exciting research subjects involving magnetic nanoparticles is their application in biological systems, including magnetic resonance imaging (MRI) or targeted drug delivery [1]. Recent reports indicate that magnetic nanoparticles such as Fe<sub>3</sub>O<sub>4</sub>, conjugated with various targeting molecules or antibodies, can be used to target specific cells *in vitro* [2]. However, the non-covalent surface modification of nanoparticles has a serious limitation for biological applications, because the exposed metal ion on the surface of nanoparticles may be cytotoxic (*in vivo* model) [3]. To make progress concerning this drawback we aim at the development of bio-compatible materials for surface coating and functionalization of nanoparticles with polymers that simultaneously bind to inorganic nanoparticles and target molecules through specific anchor groups and carry detector molecules for the detection of nanoparticles by optical methods.

Iron oxides can be synthesized and conjugated with traditional low molecular weights drugs [4] and/or ribonucleic acids (RNA) [5] which are inherently difficult to deliver due to their size and polarity. The high potential of antigen coding RNA as recombinant vaccines for prevention and therapy of infectious diseases and cancer is

limited by its fast degradation in extracellular space. Immobilization onto magnetic nanocarriers may prove to be a promising option to circumvent the degradation of vaccine RNA thereby improving its immunopharmacological properties.

## 2 EXPERIMENT

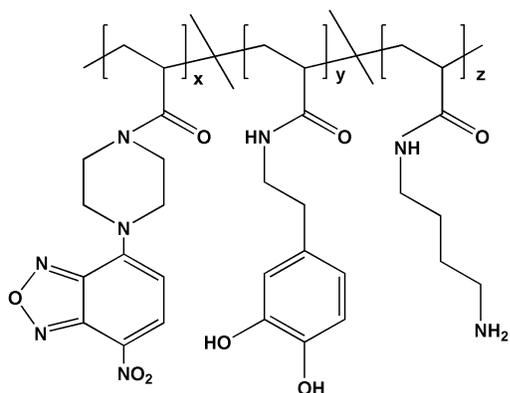
Ferrimagnetic  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles were synthesized according to ref. [6], and functionalized using a multidentate functional copolymer [5,7] carrying catecholate groups as surface binding ligands for the iron oxide nanoparticles, a fluorescent dye for optical detection and free amino groups for the attachment of poly(I:C) ligands. 15 mg of  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> was treated with 25 mg of the reactive polymer dissolved in N,N-dimethylformamide (DMF). The reaction was carried under inert atmosphere and vigorous mechanical stirring at 50 °C for 12 hours followed by cooling the reaction system to room temperature. To remove unbound polymer the coated magnetic particles in the solution were extracted by a magnetic particle concentrator (Dynal MPC1-50, Dynal Biotech, France) at room temperature. The isolated magnetic nanoparticles were washed with DMF ensuring the removal of unreacted polymer and subsequently dispersed in methyl imidazole buffer (MeIm, 0.1 M, pH 7.5). A portion of the washed magnetic particles was freeze-dried for subsequent characterization. The average crystallite size of particles with and without functional polymer coating was estimated using TEM.

dsRNA Poly(I:C) was obtained commercially (Sigma) and prepared to a final concentration of 2 mg/ml. Poly(I:C) contains phosphate groups at its 5' end which makes it susceptible to functionalization in the presence of a primary amine making use of phosphoramidate chemistry [5,8]. In a typical experiment aliquots of 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide (EDC, 0.013 M stock solution in methyl-imidazole (MeIm) buffer) and poly(I:C) were mixed. The mixture was coupled to the amine

functionality of the polymer functionalized maghemite nanoparticles. The aliquots were immediately frozen. 10  $\mu$ l of each sample were loaded into a 1% agarose gel and the run was carried out at 60 V for 1 h. All the above mentioned experiments were carried out in RNase free solutions and environment.

### 3 DISCUSSION

In this contribution we introduce a novel multifunctional polymeric ligand to immobilize dsRNA poly(I:C) onto  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles that curbs the multistep tailoring of nanoparticle functionalization. The multifunctional polymeric ligand combines three features: (i) an anchor group based on dopamine which is capable of binding to many metal oxides (e.g. Fe<sub>2</sub>O<sub>3</sub>, TiO<sub>2</sub>), [7] (ii) a fluorescent dye (as a marker) and (iii) a reactive functional group which allows binding of various biomolecules onto inorganic nanoparticles. dsRNA poly(I:C) contains a 5' end phosphate group which makes it amenable to bind to amine moieties by making use of phosphoramidate chemistry [8]. The biological activity of these poly(I:C) coated 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 was proven experimentally as sketched in Figure 1.

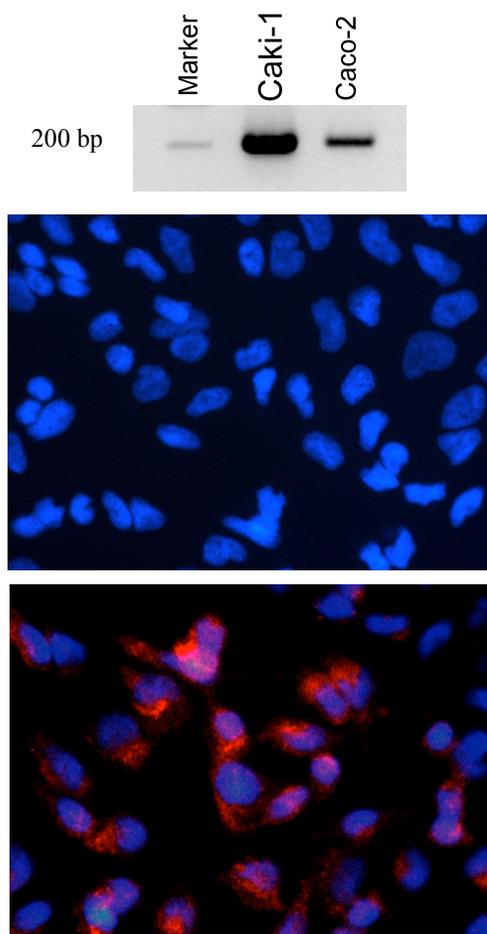


**Figure 1.** Multifunctional copolymer containing 3-hydroxytyramine (dopamine) as anchor for metal oxide binding, piperazinyl-4-chloro-7-nitrobenzofurazane (pipNBD), a fluorescent dye molecule, and functional amine group for coupling of poly (I:C).

Commercial Caki-1 cells were cultured under the conditions described in ref. [9] until reaching confluence of 95-100%. The presence of the 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 with 1% agarose. The total RNA was reverse transcribed into cDNA and subsequently amplified by RT-PCR. Subsequently, the amplified PCR products were analyzed

by 2% agarose gel electrophoresis with ethidium bromide staining along with a 100 bp DNA ladder (Figure 2, top). The 2% agarose gel showed an expected band of approximately 208 base pairs (bp). Caco-2 cells were used as a positive control of hTLR3 mRNA expression to confirm the specificity of the used primers and PCR, since it has been previously reported to express potentially functional TLR receptors like TLR3 and TLR9 [10]. By RT-PCR, we demonstrated that human TLR3 (hTLR3) is expressed in Caki-1 cells with a size of 208 base pairs.

The TLR3 expression on Caki-1 cells was visualized with an immunodetection technique (Figure 2, bottom). After a blocking step, the cells were incubated with



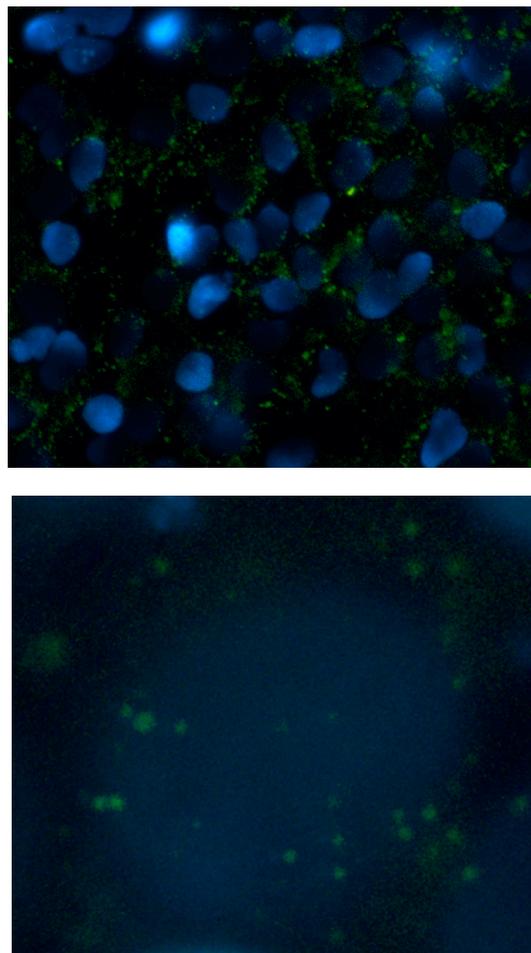
**Figure 2. Top:** Expression of TLR3 mRNA on Caki-1 and Caco-2 cells (208 base pairs). **Bottom:** Immunofluorescence for TLR3 in Caki-1 cells. (a) Pre-immune serum as used as control: no signal can be observed. (b) TLR3 monoclonal antibody was used as well as the correspondent Tex-Red fluorophore conjugated secondary antibody (red). The red fluorescence signal is clearly visualized on the Caki-1 cells. Nuclei were visualized by staining with 4,6-diamino-2-phenylindole (DAPI) (blue).

Toll-like receptor 3 mouse monoclonal antibody raised against full length hTLR3. Secondary antibody goat anti-mouse IgG-Texas Red conjugated was incubated with the Caki-1 cells, and the nuclei were stained with 4,6-diamino-2-phenylindole (DAPI) (Figure 2b, bottom). Subsequently, the cells were analyzed using optical light microscopy, using a reflected light fluorescence attachment at the emission wavelength of 456 nm and 620 nm. Controls were performed using pre-immune serum (Figure 2a, bottom). The red fluorescence provided by the secondary antibody-conjugated Texas red fluorophore signal proves the presence of TLR3 on the Caki-1 cells, in accordance with the RT-PCR results (Figure 2, top).

Figure 3 shows phase contrast microscope images which are representative of the extent of magnetic poly(I:C) coupled polymer functionalized nanoparticles surface anchoring on cells. Caki-1 cells were incubated with well dispersed poly(I:C) coupled polymer functionalized maghemite nanoparticles (37 °C, 5% CO<sub>2</sub>, 3 hrs). The cells were stained with DAPI to visualize nucleic acids (blue). Subsequently, they were analyzed using optical light microscopy with a reflected light fluorescence attachment at the emission wavelength of 456 nm and 530 nm to visualize the DAPI staining and the magnetic fluorescent maghemite nanoparticles respectively. The green fluorescence in Figure 3a and 3b shows the presence of functionalized  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> as carriers of poly(I:C) around the cell walls expressing TLR3. Controls were performed with polymer functionalized  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanocrystals in the absence of poly(I:C) under similar conditions where the nanoparticles were neither observed on the cells surface nor inside during 3 h incubation period. Nanoparticle-cell interaction depends on the surface aspects of materials that determine the cell behavior. Specific  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanocrystals were prepared, surface functionalized with a polymer and coupled to poly(I:C) for targeting cell expressed surface receptors and thereby preventing endocytosis. dsRNA functionalized nanoparticles show a high affinity for cell membrane. This opens up new opportunities for selective marking cells using magnetic properties of the nanoparticles that may be of 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 better understanding of the processes by which TLRs regulate adaptive immunity may yield not only improved treatment of infectious diseases but also the treatment and prevention of allergic and certain autoimmune disorders.

To assay cell proliferation of polymer functionalized  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanocrystals, a XTT assay was performed. Caki-1 cells were cultured in 96-well plates according to the cell culture procedure described at 3x10<sup>4</sup> cells/ well [9]. The cells were incubated with given concentrations of functionalized  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles (10, 50 and 100  $\mu$ g/mL) in triplicate for 12h. Cells were washed once with PBS and cell viability was determined using XTT (2,3-Bis(2-methoxy-4-nitro-5-sulphonyl)-2H-tetrazolium-5-

carboxanilide) [11]. Polymer derivatized nanoparticles have almost no effect on the cell proliferation when compared with the control. While concentrations of 10 and 50  $\mu$ g/ml did not harm the cells, concentrations of 100  $\mu$ g/ml caused only 15% of cell death. As the toxicity of polymer functionalized nanoparticles is very low even at higher concentrations,  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> can be used as an efficient vehicle for carrying poly(I:C)



**Figure 3.** Light microscopy images of Caki-1 cells incubated with poly(I:C) coupled  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanocrystal functionalized with multifunctional polymer carrying a fluorescent dye (green). Poly(I:C)-nanoparticles complex binds specifically to the TLR3 expressed on the Caki-1 cells (a). Higher magnification image clearly shows the nanoparticles on a single cell (b). Nuclei were visualized by staining with 4,6-diamino-2-phenylindole (DAPI) (blue).

## 4 CONCLUSIONS

In summary,  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles were surface functionalized using a novel multifunctional polymer for the selective binding of poly(IC) using phosphoramidate chemistry. The TEM and light microscopy show well

dispersed polymer functionalized  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanocrystals. The presence of TLR3 was demonstrated in the Caki-1 cell line by RT-PCR and immunostaining techniques. The dsRNA coupled nanoparticles were used to visualize the TLR3 receptors.

## ACKNOWLEDGMENTS

We are grateful to the Materials Science Center (MWFZ) of the University of Mainz for support.

## 5 REFERENCES

1. H. L. Grossman, W. R. Myers, V. J. Vreeland, R. Bruehl, M. D. Alper, C. R. Bertozzi, J. Clarke, *Proc. Natl. Acad. Sci. USA* **101**, 129 (2004).
2. J. Lange, R. Kotitz, A. Haller, L. Trahms, W. Semmler, W. Weitschies, *J. Magn. Magn. Mater.* **252**, 381 (2002).
3. C. Kirchner, T. Liedl, S. Kudera, T. Pellegrino, A. M. Javier, H. E. Gaub, S. Stolzle, N. Fertig, W. J. Parak, *Nano Lett.* **5**, 331 (2005).
4. R. Weissleder, K. Kelly, E. Y. Sun, T. Shtatland, L. Josephson, *Nat. Biotechnol.* **23**, 1418 (2005).
5. M. I. Shukoor, F. Natalio, V. Ksenofontov, M. N. Tahir, M. Eberhardt, P. Theato, H. C. Schröder, W. E. G. Müller, W. Tremel, *Small* **3**, 1374 (2007).
6. T. Hyeon, S. S. Lee, J. Park, Y. Chung, H. B. Na, *J. Am. Chem. Soc.* **123**, 12798 (2001).
7. M. N. Tahir, M. Eberhardt, P. Theato, S. Faiß, A. Janshoff, T. Gorelik, U. Kolb, W. Tremel, *Angew. Chem. Int. Ed.* **45**, 908 (2006).
8. (a) B. C. F. Chu, G. M. Wahl, L. E. Orgel, *Nucl. Acids Res.* **11**, 6513 (1983).
9. N. Glube, E. Closs, P. Langguth, *Mol. Pharmaceut.* **4**, 160 (2007).
10. A. Sato, M. Iizuka, O. Nakagomi, M. Suzuki, Y. Horie, S. Konno, F. Hirasawa, K. Sasaki, K. Shindo, S. Watanabe, *J. Gastroenterology and Hepatology* **21**, 521 (2006).
11. N. Roehm, G. H. Rodgers, S. M. Hatfield, A. L. Glasebrook, *J. Immunol. Methods* **142**, 257 (1991).