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Cell Compatible Trimethoprim-Decorated Iron Oxide Nanoparticles Bind Dihydrofolate Reductase for Magnetically Modulating Focal Adhesion of Mammalian Cells

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Supporting Information

ABSTRACT: On the basis of the high affinity binding of trimethoprim (TMP) to *Escherichia coli* dihydrofolate reductase (eDHFR), TMP-decorated iron oxide nanoparticles bind to eDHFR with high affinity and specificity, which allows magnetic modulation of focal adhesion of mammalian cells adhered to a surface. Besides being the first example of nanoparticles that selectively bind to eDHFR, the biocompatibility of the conjugate of TMP−iron oxide nanoparticles renders a convenient and versatile platform for investigating the cellular responses to specific, mechanical perturbation of proteins via a magnetic force.

Among a large variety of nanomaterials, magnetic nanoparticles[1−3] have received considerable attention in the past decade because they offer precisely controlled size, ability to respond to noncontact manipulation, and enhancement of contrast in magnetic resonance imaging (MRI). As a result, magnetic nanoparticles promise many applications in biology and medicine, including medical imaging,[4,5] protein purification,[6−8] bacteria capture,[9] and drug delivery.[10] Particularly, because of their inherent biocompatibility, iron oxide magnetic nanoparticles have become the leading candidate for developing biofunctional molecular imaging agents for targeted imaging and therapy.[4,11] Despite the considerable advances in the preparation of iron oxide magnetic nanoparticles,[1] their biofunctionalization still suffers several shortcomings. For example, it is quite common to attach poly(ethylene glycol) (PEG) on the surface of iron oxides for reducing unwanted accumulation of the nanoparticles in liver or spleen in vivo, but this attachment also decreases the binding efficiency of the nanoparticles to their targets. In principle, the link of antibodies onto the nanoparticles can increase specificity, but it is nontrivial to control the orientation and the number of antibodies on the nanoparticles. On the other hand, the decoration of the coordination complex of nickel-nitrilotriacetic acid (Ni-NTA) on magnetic nanoparticles, via a dopamine or other linkers,[6,7] has achieved high specificity and capacity. The potential cytotoxicity associated with nickel, however, prevents the direct use of Ni-NTA on live cells for long duration.[12] These limitations require the development of a new approach to present proteins on magnetic nanoparticles without compromising the binding affinity, biological compatibility, and molecular orientation of the proteins.

To satisfy the above requirements, one promising candidate is the small-molecule ligand/receptor pairs that have already gained popularity in protein pull-down assays, which imply appropriate orientation of the proteins on the surface of the particles after the binding between the ligands and the receptors. Among several possible choices of ligand/receptor pairs (e.g., biotin/avidin,[13] GSH/GST,[14] TMP/eDHFR,[15] and amylose/maltose binding protein (MBP)[16]), we chose trimethoprim (TMP) as the ligand and *E. coli* dihydrofolate reductase (eDHFR) as the receptor because of the high affinity (K_d = 9 nM) of TMP exhibited for the bacterial DHFR.[15] Although there are agarose−methotrexate microbeads, they bind to both human DHFR and bacterial DHFR indiscriminately, and methotrexate on iron oxide magnetic nanoparticles retains its cytotoxicity.[17] With respect to the compatibility/functionality with mammalian cells, trimethoprim is more advantageous because its toxicity in mammalian cells is lower than that of methotrexate. More importantly, the affinity of TMP for mammalian DHFR homologues is about 50000 times lower than for eDHFR, thus allowing selective manipulation of an ectopically expressed eDHFR fusion protein in a mammalian cell. Thus, TMP/eDHFR is considered to be a bio-orthogonal system in eukaryotic cells that has been used successfully on many occasions.[15,18] In addition, the binding between TMP and eDHFR is monovalent, which disfavors the cross-link that usually occurs in the biotin/avidin-based systems. The orthogonality, monovalency, and small size of TMP offer a tremendous advantage for TMP/eDHFR pair over many other affinity systems, thus making TMP an ideal candidate for decorating iron oxide nanoparticles for biomedical applications.

On the basis of the above rationale, we used the recently developed dopamine anchors[7,19] to attach TMP to iron oxide nanoparticles (∼6 nm in diameters) for binding eDHFR fusion proteins and manipulating cells. Our results show that TMP-functionalized nanoparticles exhibit ultrahigh affinity for eDHFR fusion proteins. This interaction is highly specific and has a long half-life, that is, behaves as an (almost) irreversible interaction under normal (nondenaturing) conditions. Most importantly, these TMP-decorated nanoparticles are cell compatible (94% cell viability after 48 h exposure to TMP decorated nanoparticles) and have the ability to alter the focal adhesion of the cells expressing the eDHFR fusion proteins in vitro. Since focal adhesion, as a fundamental cellular process, has important implications for development, differentiation, disease, and regeneration,[20] this result is significant because it offers a simple and viable process for investigating cellular responses to mechanical perturbation of specific proteins via magnetic (i.e., noncontact) modulation.

Scheme 1 shows a simple procedure to anchor TMP on an iron oxide magnetic nanoparticle. We first synthesized **1** from trimethoprim via the selective demethylation of the para ether

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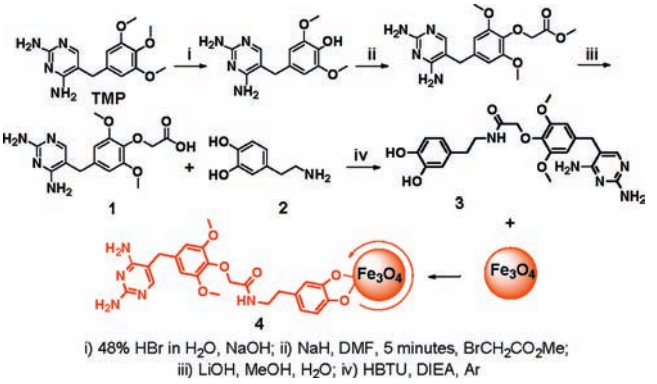
Scheme 1. Synthetic Route of the TMP-Decorated Iron Oxide Nanoparticles



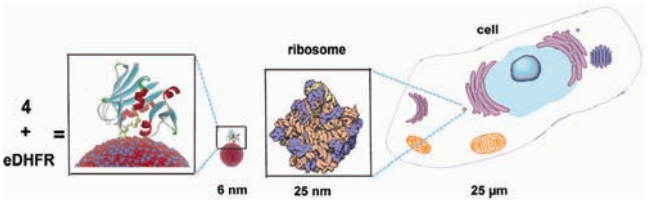


Figure 1. Possible binding of **4** with eDHFR protein on the nanoparticles; and relative size of **4**, a ribosome, and a cell.[22]

group and the subsequent attachment of a methylene carboxylate group. Then we coupled the dopamine (**2**) with **1** via HBTU and DIEA in DMF to generate **3** (yield =31%). Finally, iron oxide nanoparticles react with **3** in phosphate buffer at the pH of 6.0 to afford the TMP-functionalized iron oxide nanoparticles (**4**). Transmission electron micrograph reveals that the nanoparticles of **4** have a diameter of about 6 nm, which is similar to that of as-prepared iron oxide nanoparticles.[21] Interestingly the attachment of TMP on the iron oxide nanoparticles barely induces the aggregation of these particles in water, likely due to the hydrophilic character of the diaminopyrimidine of TMP. Besides, the IR spectra[21] of **4** exhibit a broad peak around 3340 cm^{-1} and a strong sharp peak centered at 1670 cm^{-1}, which originate from N−H (from NH_2) and C═O (from amide) stretch vibrations, respectively, thus indicating the presence of **3** on **4**. In addition, weight analysis gives the estimation of about 37 molecules of **3** on each iron oxide nanoparticle.[21]

As shown in Figure 1, a simple molecular docking model based on the lattice of magnetite[3] and the crystal structure of the complex of TMP and eDHFR[23] suggests that it is possible for **4** to bind the eDHFR protein without a spacer between dopamine and TMP. Because of the robust binding between dopamine and iron oxide,[7,19] the conjugate of **4** and eDHFR should be kinetically stable, which likely results in a relatively constant orientation of eDHFR on the surface of the nanoparticles for presenting other proteins via fusion at the (N or C) terminus of eDHFR. Most importantly, the comparable sizes between eDHFR and the nanoparticle indicate that the nanoparticles should minimize unwanted nonspecific interactions usually associated with microparticles because the target proteins can cover the surface of the nanoparticles effectively and quickly. In addition, the comparison of the diameters of **4** (6 nm), a ribosome (20−30 nm), and a cell (e.g., COS-1 cell is about

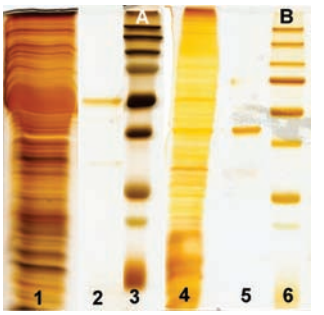


Figure 2. SDS/PAGE analysis (by silver staining[24]) of (A) binding of **4** to GFP-TEV-HA-eDHFR in a *E. coli* cell lysate: lane 1, cell lysate; lane 2, elution by 2× sds loading buffer at 60 °C for 4 h; lane 3, molecular weight marker and (B) binding of **4** to eDHFR-HA-GFP in a mammalian cell lysate: lane 4, cell lysate; lane 5, elution by 2× sds loading buffer at 60 °C for 4 h; lane 6, molecular weight marker.

20−30 μm) suggests that it is possible to use magnetic nanoparticles to manipulate cellular components (e.g., ribosomes), cellular organelles (e.g., nucleus), and cells via a magnetic force. However, to realize the magnetic control of such a wide range of targets, **4** has to meet two basic prerequisites: (i) selectively binding eDHFR fusion proteins and (ii) being cell compatible.

We first confirmed that **4** can specifically bind eDHFR fusion proteins. After incubating **4** with the lysate of *E. coli* overexpressing GFP-TEV-HA-eDHFR for 2 h in PBS (TEV is a protein sequence which recognized by tobacco etch virus protease; HA is a protein epitope derived from hemagglutinin), we used a magnet to attract nanoparticles to the wall of the vial and washed them twice with PBS, and then eluted the protein using loading buffer (LB) containing 4% sodium dodecylsulfate (SDS) at 60 °C for 4 h. As shown in Figure 2A, elution by LB at 60 °C results in one band (lane 2), which is the fusion protein GFP-TEV-HA-eDHFR (confirmed by anti-HA Western blot[21]). Meanwhile, no protein was eluted by LB at room temperature. These results confirm that **4** selectively and tightly binds to GFP-TEV-HA-eDHFR from the *E. coli* lysate. We also used the same conditions to bind the fusion protein, eDHFR-HA-GFP, expressed in a mammalian cell (HeLa). As shown in Figure 2 B, elution by LB at 60 °C gives one band (lane 5), which is eDHFR-HA-GFP. Since the HeLa cell expresses the eDHFR-HA-GFP at relatively low levels against a noisy background,[21] this result further confirms the high specificity of **4** to eDHFR. After being separated from the lysates, the conjugates, GFP-TEV-HA-eDHFR−**4**, exhibit green fluorescence,[21] confirming that the GFP proteins preserve their innate properties after being linked to the nanoparticles by fusing with eDHFR.

After confirming the binding efficiency of **4** to eDHFR, we assessed the biocompatibility of the nanoparticles toward mammalian cells. After 2 days and 5 days incubation with different amount of **4**, COS-1 and HeLa cells received a 50% trypan blue solution as a negative stain for live cells.[25] As shown in A and B of Figure 3, more than 90% of the cells are viable even after 5 days incubation with 200 μg/mL of **4**, suggesting that **4** is compatible with the cells. Furthermore, after incubating the cells with **4** for 7 days, we transfected the cells with a plasmid expressing a monomeric red fluorescence protein−glutathione-*S*-transferase fusion protein (mRFP−GST) and found that these cells successfully express mRFP−GST.[21] These results prove that cells are viable after prolonged exposure to the TMP-decorated iron oxide

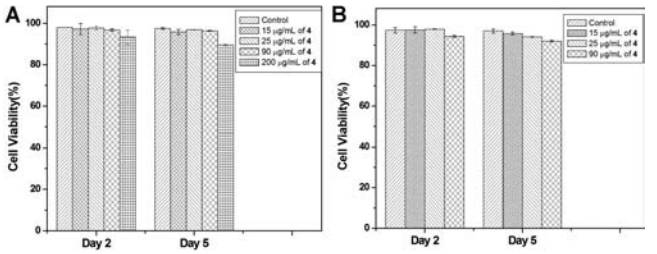


Figure 3. Viability of (A) COS-1 and (B) HeLa cells incubated with different amount of **4** (15, 25, 90, and 200 μg/mL) after 2 days and 5 days (tested by a 50% trypan blue solution).

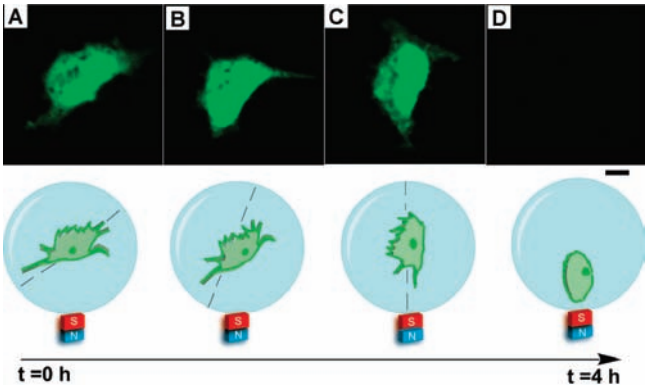


Figure 4. Magnetically modulated focal adhesion of COS-1 cell for 4 h. (A) t = 0 h (elongate forming a diagonal (45°) axis running upward from right to left), (B) t = 1.5 h (shift down slightly and the axis move to 20°), (C) t = 3 h (twist and run vertically, overall a 45° rotation), (D) t = 4 h (detached, no longer visible). The relative position of the magnet versus the cell is shown in top view (scale bar =10 μm).

nanoparticles and the uptake of **4** causes very few adverse effects to the protein expression in the cells.

Though pioneering research has been carried out by employing magnetic microbeads to study focal adhesion,[26] the corresponding investigations using nanomaterials have not been undertaken. Thus, we decided to use **4** to perturb the focal adhesion of cells. After establishing the cell compatibility of **4**, we showed that cells incubated with **4** were attracted to a magnet when trypsinized.[21] Subsequently, we incubated COS-1 cells, transiently transfected with eDHFR-HA-GFP, with 25 μg of **4** in 1 mL complete medium for overnight.[21] As shown in Figure 3A, the concentration of **4** used (25 μg) has been shown to have little effect on cell viability even after 5 days incubation. After washing the cells three times with Dulbecco's PBS to remove unbound **4**, we placed a magnet (with surface magnetic field about 4000 G) and observed the behavior (e.g., focal adhesion) of the cells over 4 h. As shown in Figure 4, the cell adheres to the plate at the initial moment, showing a characteristic asymmetric shape (nonadhered cells are typically round), that is the cell elongates along a diagonal axis running upward from left to right. After being under magnetic attraction for 1.5 h, the cell shifts down slightly so the axis of the cell tilts toward the vertical direction (Figure 4B). After 3 h attraction, the cell twists in such a way that its axis runs almost vertically (Figure 4C). After 4 h, the cell detaches from the surface and moves out of the view field (Figure 4D). Under the magnetic attraction for 4 h, the iron oxide magnetic nanoparticles in the cells clearly exert a mechanical force on the cell to cause cell-shape distortion and cell detachment from the surface. Thus, **4** permits magnetic modulation of focal adhesion of live cells. This effect is absent in a control group of cells (not incubated with **4**).[21] Given that eDHFR and GFP are not expected to associate strongly with mammalian proteins, the strong attraction of COS-1 incubated with **4** to a magnet can account for this effect.

In conclusion, we used biocompatible TMP-decorated iron oxide magnetic nanoparticles to bind eDHFR fusion proteins from cell lysate and further magnetically modulate focal adhesion of live cells. The use of nanoparticles for binding a specific protein from the milieu of a cell lysate opens many opportunities, ranging from ectopic protein delivery to magnetic manipulation of cellular functions because, unlike microbeads, nanoparticles undergo endocytosis easily. Although the mechanism of the magnetic modulation of focal adhesion has yet to be established, the further development of this approach ultimately may allow the control of the spatiotemporal behavior of proteins or subcellular organelles, which should provide a new way to help validate functional impact of the locations of proteins and contribute to establishing and regulating molecular pathways for a wide range of cellular functions.

■ ASSOCIATED CONTENT

Supporting Information. Experimental sections, NMR, LC−MS, sequence of GFP-TEV-HA-eDHFR and eDHFR-HA-GFP protein, infrared spectra, TEM, fluorescent image, SDS-PAGE analysis, anti-HA Western blot, and the video of cell migration. This material is available free of charge via the Internet at http://pubs.acs.org.

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