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Folate-Mediated Cell Targeting and Cytotoxicity Using Thermoresponsive Microgels

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One of the major concerns regarding cancer treatment is the low therapeutic index of the drugs used.[1] This limitation can be partially overcome by using drugs that are specifically targeted to cancer cells. Several classes of targeting species have been used such as sugars, lectins, vitamins, and peptides.[2−4] Cancer cells require excessive folic acid, which is a ligand for folate receptors (FRs), to enable their rapid proliferation.[1] Folic acid has very high affinity for FR (K_D = 100 pM).[1] In the past, conjugates of folic acid have been used for targeted delivery of radionuclides,[5] oligonucleotides,[6] and drugs[7] to cancer cells, wherein these conjugates enter cells via folate receptor-mediated endocytosis.[8] Here we report selective endocytosis of temperature-responsive hydrogel nanoparticles that are conjugated to folic acid. We also show that these nanoparticles reside in the cytosol following uptake and exhibit a pronounced temperature-dependent cytotoxicity.

The hydrogel nanoparticles, or microgels,[9] used in this study are mainly composed of poly(*N*-isopropylacrylamide) (pNIPAM), which exhibits a lower critical solution temperature (LCST) of ∼31 °C in water. At this temperature the nanoparticle undergoes an entropically driven transition from a swollen state to a deswollen state.[10] The hydrogel nanoparticles described in this work are of a core−shell morphology, where both the core and the shell are composed mainly of pNIPAM.[10] The core is fluorescently labeled to enable particle tracking, while the shell contains an amine comonomer to enable conjugation with folic acid. The rationale for using a core−shell morphology is to provide a higher number of amine groups on the surface of the particles and consequently a higher number of folic acid groups, which can then easily interact with the FRs on the cell. For conjugation of folic acid to the particles, we use standard carbodiimide coupling method.[11] The folate-conjugated particles undergo a phase transition at ∼32 °C and exhibit a hydrodynamic radius of ∼135 nm at 25 °C (T < LCST) and ∼50 nm at 37 °C (T > LCST) (Supporting Information).

In this study, KB cells were chosen to investigate the cellular uptake of the folate-conjugated microgels. The cells were first cultured in folate-depleted medium to induce overexpression of FRs on the cell membrane. Both the folate-conjugated microgels (AFA-FOL-171-2) and the control microgels (AFA-171-2) were incubated with KB cells, and cellular uptake was quantitated by flow cytometry. We find from these experiments that the fluorescence from AFA-FOL-171-2 incubated cells is ∼10 times higher than that measured for AFA-171-2 incubated cells (Supporting Information). The slight fluorescence observed for AFA-171-2 may be due to non-specific uptake or electrostatic binding of the polymer particles to the cells. Note that the AFA-171-2 particles possess a slightly positive surface charge due to the amine comonomer in the particle shell. The subcellular localization of the fluorescently labeled particles was confirmed by confocal microscopy of KB cells following incubation with each particle type. The fluorescence images of AFA-171-2 (Figure 1a) and AFA-FOL-171-2 (Figure 1b) suggest that only the folate positive particles are internalized by the cells. Also, the punctate fluorescence observed for AFA-FOL-171-2 (Figure 1b) may suggest that these particles are located within endosomes.

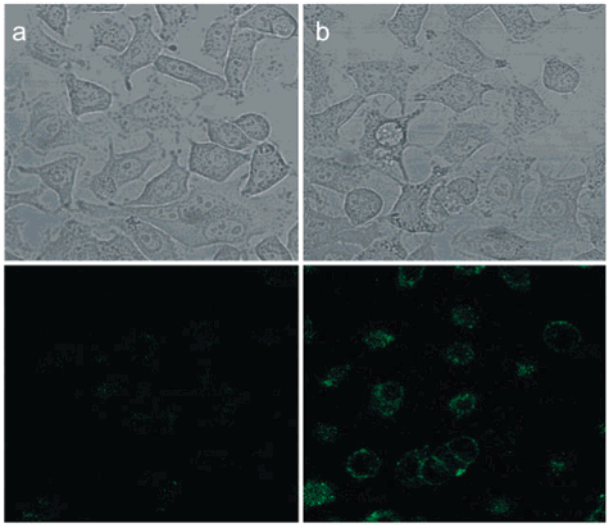

Figure 1. Optical microscopy images of treated KB cells incubated at 25 °C. Transmittance image (top) and confocal fluorescence image (bottom) for (a) AFA-171-2 and (b) AFA-FOL-171-2.

To determine if the particles internalized by the cells are indeed in endosomal compartments, a secondary dye-staining method was used to track the acidic compartments in the cells. The cells were incubated with AFA-FOL-171-2 and lysotracker red dye at 27 °C (Figure 2a) and at 37 °C (Figure 2b). It is evident that the majority of the particles lie outside of the endosomes, since the green fluorescence is largely anticorrelated with the red fluorescence channel, which marks the periphery of the endosomes. This suggests that the punctate fluorescence initially observed is not due to localization of the particles in the endosomes, but may be due to isolated particles or aggregation of the particles within the cells. Also, the observed behavior does not appear to be temperature-dependent. There is no marked difference in the fluorescence pattern when the particles were incubated at 27 °C (Figure 2a), which is below LCST of the particles, as compared to incubation at 37 °C (Figure 2b), which is above LCST and where the particles are more hydrophobic. This observed behavior of the particles being endocytosed but not being retained in the endosomes greatly increases the potential applicability of these materials for cytosolic drug delivery. Similar behavior has been observed previously for block copolymer micelles.[12] For cytosolic delivery to be efficient, any drug−folate conjugate must be released into the cytosol following

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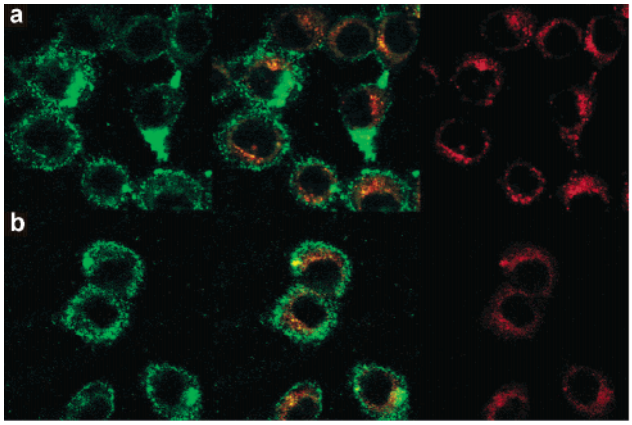

Figure 2. Cellular localization of AFA-FOL-171-2 and Lysotracker red dye within KB cells. (a) At 27 °C. Left, fluorescein channel; right, Lysotracker red channel; center, overlap of the two channels. (b) At 37 °C. Left, fluorescein channel; right, Lysotracker red channel; center, overlap of the two channels.

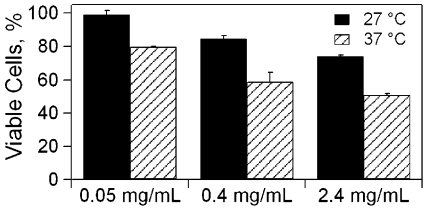

Figure 3. KB cell viability at varying concentrations of AFA-FOL-171-2 at 27 °C and 37 °C after 4 h incubation.

endocytosis.[13] For most particulate carriers, it is generally assumed that in addition to the presence of a targeting ligand, a secondary triggering mechanism must occur in the endosome to release the drug in the cytosol.[14−16] Most of these mechanisms are based on a drop in endosomal pH. Interestingly, our hydrogel nanoparticles lack any purposely designed secondary mechanism and yet are efficiently delivered into the cytosol after being endocytosed. Hence, these structures have potential to be used as carriers for cytosolic delivery of drugs to cells that overexpress FRs.

Particle-induced cytotoxicity was investigated to evaluate the impact of uptake on cell viability. Cytotoxicity was determined as a function of concentration of the polymer particles and incubation temperature by a standard MTT cell viability assay. Figure 3 shows that cytotoxicity increases with an increase in the concentration of the polymer at 27 °C, but for each concentration there is a marked decrease in viable cells at 37 °C. Thus, a simple temperature switch induces a greater degree of cell death following particle uptake. Since there are no obvious chemical inhibitors of cellular metabolism in the polymer structure, the cytotoxicity may be of some physical origin. For example, pNIPAM hydrogel nanoparticles become relatively hydrophobic at temperatures above their LCST and can aggregate in aqueous media as a result. The presence of residual anionic charges from the initiator used during the polymerization typically prevents this aggregation.[9] However, in high ionic strength solutions such as cell growth media, these charges can be shielded and the particles can aggregate at high temperature.[17] We therefore tentatively attribute the observed temperature-dependent cytotoxicity to particle aggregation in the cytosol. It is further known that pNIPAM at high temperatures causes protein adsorption and denaturation, which is also most likely associated with particle aggregation in the cytosol.[18]

Folate-conjugated hydrogel nanoparticles provide a novel model system for specific targeting of cancer cells. The ability of the particles to escape from the endosome into the cytosol, in addition to their large swelling capacities, makes them potential candidates for cytosolic delivery of drugs. Also, the temperature-dependent cytotoxicity suggests a route to cell-specific, externally triggered, nonpharmacological antitumor activity. The temperature at which this cytotoxicity is observed (37 °C) is not necessarily physiologically useful, and hence, further work is required to shift the induced cytotoxicity to slightly higher temperatures; this is a current focus of our efforts.

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Supporting Information Available: Variation of the size of the folate-conjugated particles as a function of temperature, flow cytometry results, and the Experimental Section. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Leamon, C. P.; Low, P. S. *Drug Discovery Today* **2001**, *6*, 44−51.
(2) Bies, C.; Lehr, C.-M.; Woodley, J. F. *Adv. Drug Delivery Rev.* **2004**, *56*, 425−435.
(3) Sudimack, J.; Lee, R. J. *Adv. Drug Delivery Rev.* **2000**, *41*, 147−162.
(4) Tkachenko, A. G.; Xie, H.; Coleman, D.; Glomm, W.; Ryan, J.; Anderson, M. F.; Franzen, S.; Feldheim, D. L. *J. Am. Chem. Soc.* **2003**, *125*, 4700−4701.
(5) Wang, S.; Luo, J.; Lantrip, D. A.; Waters, D. J.; Mathias, C. J.; Green, M. A.; Fuchs, P. L.; Low, P. S. *Bioconjugate Chem.* **1997**, *8*, 673−679.
(6) Leamon, C. P.; Cooper, S. R.; Hardee, G. E. *Bioconjugate Chem.* **2003**, *14*, 738−747.
(7) Lee, R. J.; Low, P. S. *Biochim. Biophys. Acta: Biomembr.* **1995**, *1233*, 134−144.
(8) Anderson, R. G. W.; Kamen, B. A.; Rothberg, K. G.; Lacey, S. W. *Science* **1992**, *255*, 410−411.
(9) Pelton, R. *Adv. Colloid Interface Sci.* **2000**, *85*, 1−33.
(10) Jones, C. D.; Lyon, L. A. *Macromolecules* **2000**, *33*, 8301−8306.
(11) Leamon, C. P.; Low, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5572−5576.
(12) Savic, R.; Luo, L.; Eisenberg, A.; Maysinger, D. *Science* **2003**, *300*, 615−618.
(13) Leamon, C. P.; Low, P. S. *J. Biol. Chem.* **1992**, *267*, 24966−24971.
(14) Vogel, K.; Wang, S.; Lee, R. J.; Chmielewski, J.; Low, P. S. *J. Am. Chem. Soc.* **1996**, *118*, 1581−1586.
(15) Rui, Y. J.; Wang, S.; Low, P. S.; Thompson, D. H. *J. Am. Chem. Soc.* **1998**, *120*, 11213−11218.
(16) Lackey, C. A.; Press, O. W.; Hoffman, A. S.; Stayton, P. S. *Bioconjugate Chem.* **2002**, *13*, 996−1001.
(17) Duracher, D.; Sauzedde, F.; Elaissari, A.; Pichot, C.; Nabzar, L. *Colloid Polym. Sci.* **1998**, *276*, 920−929.
(18) Kawaguchi, H.; Fujimoto, K.; Mizuhara, Y. *Colloid Polym. Sci.* **1992**, *270*, 53−57.

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