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Multifunctional Gold Nanoparticle−Peptide Complexes for Nuclear Targeting

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Targeted entry into cells is an increasingly important area of research. The nucleus is a desirable target because the genetic information of the cell and transcription machinery reside there. The diagnoses of disease phenotype, the identification of potential drug candidates, and the treatment of disease by novel methods such as antisense therapy would be enhanced greatly by the efficient transport of materials to living cell nuclei.[1]

Targeted nuclear delivery is a challenging task, as any nuclear probe must satisfy minimally the following requirements: it must (i) enter the cell, via receptor-mediated endocytosis (RME), for example; (ii) escape endosomal/lysosomal pathways; (iii) possess a nuclear localization signal (NLS) to interact with the nuclear pore complex; and (iv) be small enough (<30 nm) to cross the nuclear membrane. Herein, results of intracellular trafficking studies of nanoparticles designed to perform all of these functions are reported.

Metal, semiconductor, polymer, and magnetic particles have been introduced into cells previously.[2] Most of these studies concerned particle entry into the cytoplasm exclusively. However, in pioneering work, Feldherr and co-workers studied nuclear translocation of gold nanoparticles carrying peptides from the SV-40 large T antigen.[2e] Their work was performed using microinjection or chemically modified cells, thus bypassing cellular membrane entry. Weissleder and co-workers derivatized superparamagnetic nanoparticles with the HIV Tat peptide.[2b] Tat is a generic membrane translocation peptide, and, when coordinated to magnetic nanoparticles, efficient nuclear targeting was afforded for the HeLa cell line investigated.

Recognizing the multitude of challenges associated with nuclear targeting, and following the presumption that no single peptide will be effective at *specific* cell recognition and nuclear targeting, we have been pursuing a new approach to nuclear translocation. The general strategy is based upon nuclear targeting in biological systems. The most efficient nuclear targeting in biology is accomplished by viruses, which commonly utilize different peptides for crossing each cellular membrane barrier. Our strategy has thus been to identify the most capable peptides for accomplishing each of the tasks stated above and then to combine these peptides on a single nanometer-sized platform. The platform chosen was a 20 nm diameter gold particle. The gold particle was modified with a shell of bovine serum albumin (BSA) conjugated to various cellular targeting peptides (Table 1). Of particular concern in the present study was whether multiple short peptide sequences could be found which, when attached to a nanoparticle core in various combinations, could satisfy or even enhance the function of one longer polypeptide.

Peptide−BSA−gold nanoparticle complexes were prepared as described previously[3] and characterized with dynamic light scattering (DLS), gel electrophoresis, and fluorescence spectroscopy. DLS revealed that BSA−peptide conjugates add <2 nm to the radius of the nanoparticle complex. The fact that BSA does not add greatly to the size of the gold particle is important in its use in constructing nuclear targeting vectors because the diameter of the nuclear pore complex is 20−50 nm depending on the cell line. The 20 nm gold particles used in this study have a maximum diameter of 25 nm when complexed with any of the BSA−peptide conjugates studied (Supporting Information). Gel electrophoresis was used to quantitate the peptide:BSA ratio (Table 1), and fluorescence spectroscopy was used to determine the number of BSA−peptide conjugates attached to gold nanoparticles (160 ± 8 for 20 nm diameter particles). Fluorescence spectroscopy also showed that when two types of peptide−BSA conjugates are mixed in aqueous solution with nanoparticles, the mole fraction, X, of BSA bound to the nanoparticles reflects X of the two BSAs added to solution (Supporting Information).

Table 1. Peptide Sequences Used in Nanoparticle−BSA−Peptide Complexes[a]

#	peptide sequence	origin of peptide	peptide/BSA
1	CGGGPKKKRKVGG	SV40 large T NLS	7 ± 1
2	CGGFSTSLRARKA	adenoviral NLS	8 ± 1
3	CKKKKKKSEDEYPYVPN	adenoviral RME	9 ± 2
4	CKKKKKKKSEDEYPYVP-NFSTSLRARKA	adenoviral fiber protein	6 ± 2

[a] Peptides were conjugated to BSA with a 3-maleimido benzoic acid *N*-hydroxysuccinimide ester linker.[4,5] Gel electrophoresis (SDS−PAGE) and fluorescence spectroscopy were used to quantify the peptide:BSA ratio (Supporting Information).

A number of techniques have been used previously to determine cellular trajectories of nanoparticles. Indeed, the use of electron microscopy with colloidal gold stains was perhaps the first modern method of cell structure characterization.[3] More recently, fluorescence microscopy has been used to locate fluorophores, including luminescent CdSe nanoparticles, inside cells.[2c] We have exploited the enormous visible light extinctions of gold nanoparticles to monitor their trajectories inside cells using a combination of video-enhanced color (VEC) microscopy and differential interference contrast microscopy[4] (DIC) (Figure 1).

In the present study, nanoparticles complexed to peptides were explored to achieve nuclear targeting in intact HepG2 cells. HepG2 cells were chosen for this study because of the known difficulty of membrane translocation in this cell line as opposed to HeLa cell lines.[5] As a test case, nanoparticle complexes with peptide #1 were introduced into the growth medium of HepG2 cells. Feldherr has shown that peptide #1, the NLS from SV-40 virus, is translocated to the nucleus when attached to gold nanoparticles, if the particles are injected into the cytoplasm.[2e] Surprisingly, when these complexes were added to cell growth media, they were found inside the cytoplasm of HepG2 cells; however, they did not enter the nucleus. Control experiments at 4 °C indicated that cell entry was via an energy-dependent pathway. This observation suggests that peptide #1 entered the cell by receptor-mediated endocytosis, but was unable to escape the endosome and target the nucleus. These results highlight the challenges associated with nuclear targeting:

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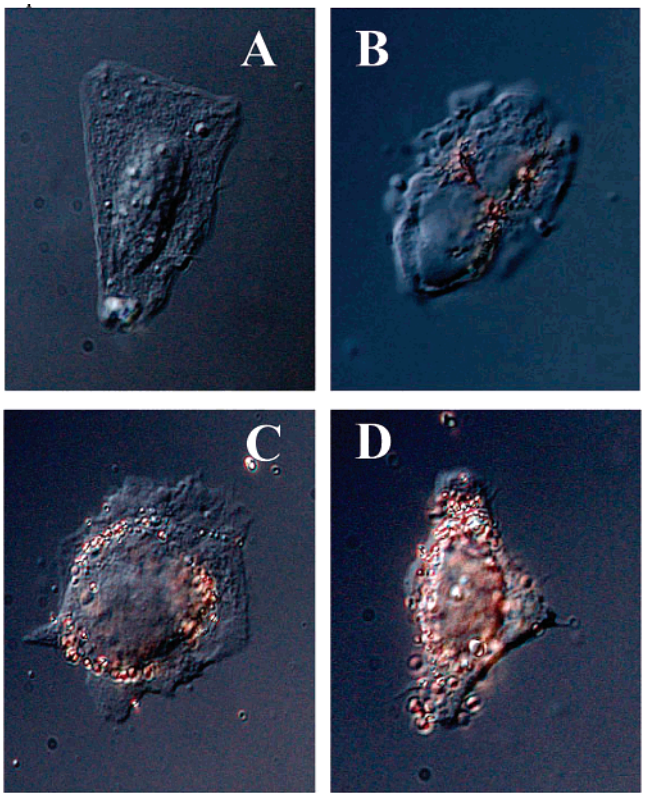

Figure 1. Nanoparticle−peptide complexes incubated with HepG2 cells for 2 h: #2 (A), #3 (B), #4 (C), and #2/#3 (D). The HepG2 cell line was obtained from the American Type Culture Collection (Rockville, MD), maintained in EMEM medium (BioWhittaker) with 10% FBS at 37 °C in a controlled CO_2 atmosphere, and incubated with nanoparticle delivery vectors for 2 h, after which time cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and then rehydrated in PBS. Once all of the cells were fixed, the cover slips with the cells were mounted onto glass slides with FluorSave mounting media (Calbiochem, CA) and then examined using a Leica DMLB DIC equipped microscope with 100X/1.3 NA objective. Images were taken and processed with a Nikon DMX-1200 digital color CCD camera. The efficiency of nuclear targeting in experiment (D) was estimated by examining more than 200 cells, of which 80% contained nanoparticles in the nucleus.

although a known NLS peptide is able to enter HepG2 cells, it cannot target the nucleus unless it is capable of endosomal escape.

To improve nuclear targeting efficiency in HepG2 cells, peptides from the adenovirus were explored. This protein is known to contain both RME and NLS sequences (#2 and #3, in Table 1). The full length fiber containing both the RME and the NLS is peptide #4 in Table 1. A comparison of the functions of these targeting peptides when complexed to a gold nanoparticle is shown in Figure 1. Figure 1A shows that peptide #2 did not enter the cell. Peptide #3 entered the cell (B), but remained trapped in endosomes and did not reach the nucleus. Peptide #4 targeted the nucleus (C); however, nanoparticles carrying peptides #2 and #3 had a greater propensity for nuclear targeting than any other single peptide explored (D).

The nanoparticle complex must present both RME and NLS peptides to enter intact HepG2 cells *and* achieve nuclear localization. The VEC-DIC results show nuclear targeting of nanoparticles modified with adenovirus NLS-RME (C) and more efficient nuclear targeting of the nanoparticle carrying adenovirus RME and adenovirus NLS as separate pieces (D). The origin of the higher nuclear targeting efficiency in nanoparticles carrying two short peptides versus one long sequence could be structural or spatial. IR spectroscopy revealed that all peptides were in an extended conformation.[6] Thus, when two short peptides are attached to a single nanoparticle, it is likely that the individual targeting signals are more accessible to cellular receptors.

Finally, although gold is an inert metal, its effect on normal cell proliferation cannot be underestimated. To determine the viability of HepG2 cells following gold nanoparticle−peptide complex uptake, an LDH colorimetric toxicity assay was performed (Supporting Information). Viability of HepG2 cells after 12 h in the presence of nanoparticle−peptide complexes was only slightly compromised (<5%) as compared to that of a control batch.

The methods used here provide a means of rapidly assessing the efficacy of various combinations of targeting peptides using nanoparticle complexes for nuclear targeting. The VEC-DIC combination microscopy permits examination of hundreds of samples per day, an improvement over costly and time-consuming electron and confocal microscopy techniques. Moreover, gold particle optical properties enable the observation of individual particles by VEC-DIC[2b] and are not susceptible to photobleaching. The multipeptide approach demonstrated using adenoviral targeting sequences provides an important test of the function of individual peptide sequences that may help increase the effectiveness of specific cell and nuclear targeting.

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Supporting Information Available: Dynamic light scattering, microscopy images for #1, LDH colorimetric viability assay, and fluorescence characterization of peptide−BSA−gold nanoparticle complexes (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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