Supramolecular Host–Guest Chemistry-Based Folate/Riboflavin Functionalization and Cancer Cell Labeling of Nanoparticles

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Supporting Information

ABSTRACT: Nanoparticle-based cellular probes are commonly designed via covalent conjugation with affinity biomolecules. Those nanobioconjugates selectively interact with cell surface receptors and induce endocytosis followed by intracellular trafficking. However, this approach requires functional modification of biomolecules that may alter their biochemical activity. Here, we show that supramolecular host–guest chemistry can be utilized as an alternative approach in nanoparticle functionalization and selective cell labeling. We have used cyclodextrin-conjugated quantum dots (QDs) for supramolecular host–guest interaction-based functionalization with folate (QD-folate) and riboflavin (QD-riboflavin), where cyclodextrin acts as a host for the folate/riboflavin guest. We demonstrate that QD-folate and QD-riboflavin selectively label cells that have over-expressed folate/riboflavin receptors and induce the endocytosis pathway similar to covalently conjugated folate/riboflavin-based nanoparticles. However, labeling is highly sensitive to the molar ratio of folate/riboflavin to cyclodextrin and incubation time. The presented functionalization/labeling approach is unique as it does not require covalent conjugation and may be extended for in vivo targeting application via simultaneous delivery of host and guest molecules.

INTRODUCTION

Nanoparticles are widely used as imaging probes, drug delivery carriers, single-molecule tracking probes and theranostic agents. In these applications, nanoparticles are transformed into functional nanoparticles or nanobioconjugates for selective targeting/labeling of tissues, cells, and biomolecules. Usual functionalization approaches involve covalent conjugation of nanoparticles with affinity biomolecules such as vitamin, oligonucleotides, aptamers, peptides, antibodies, and carbohydrates. Variety of bioconjugation reagents along with conjugation protocols are now commercially available, which are routinely exploited in deriving functional nanoparticles. However, there are few limitations in such a covalent conjugation approach that include loss of biochemical activity of affinity biomolecules because of covalent conjugation, specialized chemistry involved in each type of functionalization, and difficulty in purification of functionalized nanoprobes. Thus, research has been directed toward advanced and alternative approaches of functionalization.

Supramolecular host–guest interaction can be viewed as a unique alternative for functionalization as it does not involve any covalent modification. In the host–guest interaction, the hydrophobic cavity of cyclodextrin (CD), cucurbituril, and calixarene can incorporate guest molecules (or a part of guest molecules) via noncovalent and weak interactions. Such host–guest interaction has been successfully utilized for functionalization of 2D surfaces, nanoparticles, cell membranes, and polymers/dendrimers. Moreover, such type of functional materials have been used in drug delivery, biosensing, and other biomedical applications. However, in all these host–guest approaches, only selected host–guest molecules (e.g. CD–adamantane, and CD–ferrocene) are used to produce stronger interactions. In reality, many of the host–guest interactions are weak/reversible, unstable in the presence of competitor molecules and difficult to be utilized for biomedical applications. In particular, the reversible host–guest interaction limits functionalization of polymers/nanoparticles with vitamins/carbohydrates/peptides and shrinks the scope for selective targeting/labeling applications under a complex bioenvironment. Here, we demonstrate that the reversible host–guest interaction between the CD host and the folate/riboflavin guest can be exploited for the functionalization of nanoparticles and targeting cancer cells followed by cellular endocytosis and subcellular trafficking. It is known that folate and riboflavin receptors are over-expressed in several types of cancer cells and their covalent conjugates with polymers/nanoparticles/CD are used for targeting cancer cells. However, the host–guest interaction-based functionalization of folate/riboflavins is not extensively utilized in cell targeting, except in one report and without details of the uptake mechanism. This is because of weaker host–guest interaction as compared to CD–adaman-
Here, we show that quantum dots (QDs) functionalized with folate/riboflavin via the host–guest interaction can successfully label cells that have over-expressed folate/riboflavin receptors and induce the endocytosis pathway similar to nanoprobes that have covalently conjugated folate/riboflavin. However, labeling is highly sensitive to the ratio of folate/riboflavin to CD and incubation time.

### RESULTS

#### Synthesis of CD-Functionalized QDs (QD(CD)_{70})

We have synthesized β-CD-functionalized QDs with an average number of 70 CDs per QD (i.e. QD(CD)_{70}) and used them in deriving folate-/riboflavin-functionalized QDs (Scheme 1). Hydrophobic QDs are transformed into polyacrylate-coated hydrophilic QDs with an average of 100 primary amine groups per particle. Next, QD(NH2)_{100} is covalently conjugated with β-CD-NH2 to produce QD(CD)_{70} that has ~70 average number of CDs per particle. Colloidal QD(CD)_{70} is incubated with varied concentrations of folate/riboflavin to produce folate-/riboflavin-functionalized QDs (i.e. QD(folate)_x and QD(riboflavin)_x), where "x" denotes the average number of folate/riboflavin per particle in the range of 1–35.

### Scheme 1. Synthetic Approach for the Preparation of QD(CD)_{70} from QD(NH2)_{100} and Transformation into Folate-/Riboflavin-Functionalized QDs via the Host–Guest Interaction

**Figure 1.** (a) UV–visible and fluorescence spectra of QD(NH2)_{100} and QD(CD)_{70}. Inset shows the colloidal solution of QD(CD)_{70} under normal (left) and UV light (right). (b) TEM image of QD(CD)_{70} showing the inorganic QD core of 4–5 nm size. (c) Hydrodynamic size of QD(NH2)_{100} and QD(CD)_{70}. (d) GPC-based determination of molecular weights of QD(NH2)_{100} and QD(CD)_{70} showing that the molecular weight increases by 90 kDa after functionalization with CD.
amines are estimated via the fluorescence test; the QD concentration is determined from their respective molar ratios to minimize particle cross-linking. The primary amines are estimated via the fluorescamine test; the QD concentration is determined from their respective molar extinction coefficient and the QDs are designated as QD(NH$_2$)$_{100}$ as the number of primary amines per QD is ~100 (Supporting Information, Figure S1). Next, QD(NH$_2$)$_{100}$ is transformed into CD-functionalized QDs by conjugating β-CD-NH$_2$ using ethylene glycol-bis(succinic acid-N-hydroxysuccinimide ester) (NHS-PEG-NHS) as the conjugation reagent. Typically, β-CD-NH$_2$ and NHS-PEG-NHS are mixed in 1:1 molar ratio for reaction of the primary amine of CD-NH$_2$ with one NHS of NHS-PEG-NHS. Next, the QD(NH$_2$)$_{100}$ solution is mixed with the excesses (10 times of primary amines present in the QDs) of this solution and under this condition other NHS of NHS-PEG-NHS react with primary amines of QDs. Next, excesses of reagents are removed via dialysis (molecular weight cut-off: 12 kDa) against fresh water. The amount of QD-bound CD is estimated by the anthrone test, and the number of CD per QD is estimated to be ~70 and designated as QD(CD)$_{70}$ (Figure 1 and Supporting Information, Figure S1). This number of CD per QD has been further confirmed from the gel permeation chromatography (GPC)-based molecular weight study and the fluorescamine test of QD(NH$_2$)$_{100}$ and QD(CD)$_{70}$ (Figure 1). The GPC results show that the molecular weight is increased by 90 kDa after the transformation of QD(NH$_2$)$_{100}$ to QD(CD)$_{70}$ which corresponds to ~70 CD. Estimation of primary amines (via fluorescamine test) in QD(NH$_2$)$_{100}$ and QD(CD)$_{70}$ also corroborates this value (Supporting Information, Figure S1). The transmission electron microscopy (TEM) image of QD(CD)$_{70}$ shows that the QD core is of 4–5 nm size, the dynamic light scattering (DLS) study shows 15–25 nm hydrodynamic size, and the zeta potential data show ~10 to ~20 mV charge at pH 7.4 (Figure 1).

**Figure 2.** (i) Evidence of the host–guest interaction of folic acid with β-CD (a) and QD(CD)$_{70}$ (b). Same concentration of folic acid is incubated with β-CD-NH$_2$/QD(CD)$_{70}$ where the molar ratio of folic acid to CD is maintained as 1:20, and then, the absorption/emission spectra of folic acid is measured. Results show that the host–guest interaction leads to increased absorbance at 360 and 280 nm and increased emission spectra at 455 nm. For the QD(CD)$_{70}$ sample, QDs are dissolved by HCl followed by neutralization with a base prior to spectral measurements. (ii) Evidence of the host–guest interaction of riboflavin with β-CD (a) and QD(CD)$_{70}$ (b) Same concentration of riboflavin is incubated with β-CD-NH$_2$/QD(CD)$_{70}$ where the molar ratio of riboflavin to CD is maintained as 1:30, and then, the absorption spectra of riboflavin is measured.
folate/riboflavin (0.04–1.4 μM); we can assume that most of the folate/riboflavin bounds with CD and the molar ratios of CD to folate/riboflavin provide the tentative number of folate/riboflavin bound to each QD. In addition, the folate/riboflavin concentration in human blood ranges from 0.040 to 0.150 μM, and thus, we may assume a similar situation under in vitro/in vivo conditions.

We have investigated that the fluorescence property of QDs remains stable in the presence of different biological substituents (Supporting Information, Table S1 and Figure S3). In addition, we have investigated the effect of co-existing substances in a complex biological environment and solution pH on the assembly of the host–guest complex. An insignificant change in the fluorescence intensity suggests that the host-guest complex is stable under a complex bioenvironment (Supporting Information, Table S1 and Figure S4).

Selective Labeling of Cancer Cells Using Folate-/Riboflavin-Functionalized QDs Prepared via Host–Guest Chemistry. We have extensively studied the labeling performance of folate-/riboflavin-functionalized QDs to cancer cells. We have selected KB cells that over-express folate receptors as well as riboflavin receptors and A431 cells that over-express riboflavin receptors. In addition, we have used Chinese hamster ovary (CHO) cells as control cells that do not over-express folate or riboflavin receptors. The results are summarized in Figures 3–7 and Supporting Information Figures S5–S9, which concludes that folate-functionalized QDs can selectively label KB cells and riboflavin-functionalized QDs can selectively label A431 and KB cells. To confirm labeling selectivity, we have performed two control experiments. First, QD(NH2)100 and QD(CD)70 are used to label KB/A431 cells, and the results show that they cannot label cells (Supporting Information, Figure S5). This result is expected as the surface chemistry is appropriately designed with extensive pegylation and overall anionic surface charge. This result also indicates that folate/riboflavin functionalization is necessary for cell labeling. Second, folate-/riboflavin-functionalized QDs are used to label CHO cells that do not have over-expressed folate/riboflavin receptors. The results show that CHO cells are not labeled by QD-folate or QD-riboflavin, indicating that the uptake of QD requires folate/riboflavin receptors on the cell surface (Supporting Information, Figures S6 and S7). In addition, two other control experiments are performed where KB cells are incubated with a mixture of folate and QD(NH2)100 or incubated with folate-functionalized QDs prepared from γ-CD-functionalized QDs (Supporting Information, Figure S8). In all cases, insignificant cell labeling are observed suggesting that nonspecific binding of folate with QD(NH2)100 or poorly interacting γ-CD host are unable to label cells.

Systematic labeling study shows that there are two critical conditions for cell labeling. First, the labeling is highly sensitive to the molar ratio of QD-bound CD to folate/riboflavin (Figures 3 and 4 and Supporting Information, Figure S9). We

Figure 3. Folate concentration-dependent labeling of KB cells by QD(CD)70. Colloidal QD(CD)70 is incubated with varied concentrations of folate, where the molar ratio of QD-bound CD to folate is kept at 1 (a), 2 (b), 5 (c), 10 (d), 20 (e), 40 (f), and 70 (g). Next, the cells are incubated with QD samples for 3 h, and the washed cells are used for bright field (BF) or fluorescence (F) imaging. (h) Flow cytometry-based quantitative estimation of QD uptake. About 20 000 cells are used for each experiment, and mean ± SD represents three independent cell-culture replicates (n = 3). Results show that labeling performance is best for the CD to folate molar ratio of 20. Scale bars are 50 μm (main figure) and 25 μm (inset).
have used different sets of nanoprobes with the molar ratio of QD-bound CD to folate/riboflavin and found that maximum labeling occurs for the ratio of 20:1 (for folate) or 30:1 (for riboflavin). Under this condition, each QD is expected to bind with 3 to 4 number of folate/riboflavin (i.e. multivalency of 3 or 4). Labeling becomes inefficient as this molar ratio is either increased or decreased. Second, labeling and subcellular trafficking is highly sensitive to incubation time and continuous incubation for longer time is essential for efficient labeling (Figure 5 and Supporting Information, Figure S10). For example, cellular uptake and subcellular localization of QD are observed under continuous incubation for 1−9 h. By contrast, if shorter incubation time is used followed by further cell growth in fresh culture media, initially labeled QDs detach from the cell surface (Figure 5).

We have quantitatively estimated the cell labeling of QD nanoprobes via flow cytometry. The cells were incubated with QD samples and labeling of QDs is quantified using the fluorescence property of QDs (Figure 3). The results clearly show that labeling is sensitive to the molar ratio of QD-bound CD to folate/riboflavin and highest for the molar ratio of 20/30 as shown in Figures 3 and 4.

We have also investigated that addition of folic acid in right concentrations into cell culture media leads to the labeling of KB cells by QD(CD)$_{30}$ which is otherwise not possible (Supporting Information, Figure S11). This labeling indicates host−guest complexation followed by cell labeling occurring under a complex biological environment.

In another control experiment, we have replaced CdSe/ZnS by Mn doped ZnS (Mn−ZnS) and prepared riboflavin-functionalized Mn−ZnS via host−guest chemistry. In this system, Mn−ZnS and riboflavin can be tracked simultaneously, as they have different excitation/emission. Labeled cells are imaged under blue excitation for riboflavin imaging and imaged under UV excitation for Mn−ZnS imaging and result show that Mn−ZnS and riboflavin are co-localized. This result indicates that the host−guest complex allows the entry of both the nanoparticle host and the riboflavin guest into cells (Supporting Information, Figure S12).

**Lipid-Raft Endocytosis and Subcellular Trafficking of Folate-/Riboﬂavin-Functionalized QDs.** We have further investigated the uptake mechanism of QDs using different endocytosis inhibitors. Cells are incubated with folate/riboflavin free culture medium and inhibitors are added in a required amount. Then, QD samples are added and incubated for another 1.5 h, and washed cells are used for flow cytometry-based quantification of QD uptake. The results show that uptake of QDs differs in the presence of inhibitors. In particular, the uptake of QD-folate and QD-riboflavin is significantly inhibited by methyl−β-CD (MBCD) and partially inhibited by sucrose (Figure 6). The results clearly suggest that the uptake of nanoprobes occurs predominantly via lipid-raft and partially via clathrin-mediated endocytosis.

We have also observed distinct subcellular localization of QD-folate and QD-riboflavin (Figure 7 and Supporting Information, Figures S13−S15). When QD-folate/QD-riboflavin is incubated with KB/A431 cells for 9−10 h, they are
Localized in the perinuclear region and specifically concentrated at one side of the nucleus (Figure 7). High magnification fluorescence images of cells labeled with both nuclear probes and QD-riboflavin and at different Z planes show that QDs localize at the same plane of the nucleus (Supporting Information, Figure S13). The colocalization study with LysoTracker and Golgi tracker shows that QD-folate/QD-riboflavin partially localizes at the Golgi apparatus and lysozome (Supporting Information, Figures S14 and S15).

It is well-known that the endocytosis uptake mechanism dictates subcellular trafficking of foreign materials. Clathrin-mediated endocytosis usually traffics them to acidic lysosomal/endosomal compartments but caveolae or lipid-raft mediated endocytosis usually traffics them toward the nucleus, endoplasmic reticulum, and Golgi apparatus. In particular, covalently attached folate-functionalized nanoprobes are reported to enter into cells via both clathrin- and caveolae-mediated endocytosis. Similarly, riboflavin-functionalized rhodamine is shown to enter into cells via both caveolae- and clathrin-mediated endocytosis. We have recently shown that the zwitterionic–lipophilic surface chemistry and <10 multivalency of nanoprobes can minimize clathrin-mediated endocytosis and induce predominant caveolae- or lipid-raft endocytosis. In the present case, predominate lipid-raft endocytosis can be explained because of the zwitterionic surface chemistry and 3/4 multivalency of folate-riboflavin-functionalized QDs that are prepared via the host–guest interaction. This predominate lipid-raft endocytosis ensures their significant trafficking toward the nucleus and Golgi apparatus. To show that these nanoprobes are less toxic, we performed conventional methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay-based cytotoxicity study (Supporting Information, Figure S16). The results show that our nanoprobes are less toxic in the concentration range used for all experiments.

On the basis of our results, we propose the cellular uptake mechanism of folate-/riboflavin-functionalized nanoprobes that is prepared via the host–guest interaction (Scheme 2). At lower folate/riboflavin concentrations, all of them forms inclusion complexes with CD, directs the QD interaction with folate/riboflavin receptors at the cell surface, induces endocytosis of QDs, and trafficks QD toward the nucleus/Golgi apparatus. At higher folate/riboflavin concentrations, their fraction is free for competitive interactions with folate/riboflavin receptors at the cell surface that restricts the QD endocytosis. Noncovalent/modular interaction of folate/riboflavin with CD helps the easier replacement of their position from CD to folate/riboflavin receptors. It is well-established that the hydrophobic cavity of CD encapsulates the hydrophobic phenyl ring of folate and the xylene part of the riboflavin. The remaining parts of folate/riboflavin stay outside of the CD cavity. Also, it is reported that folate and riboflavin can interact with the receptor proteins through the guanidine, hydroxyl groups, and other hydrophobic groups as well. Considering the comparable binding constant (≈10^4 M^-1) of CD with riboflavin and tryptophan (through which the riboflavin receptor binds with riboflavin), it may be assumed that the receptors first bind with the parts of folate/riboflavin which are outside the CD.
cancer cells followed by cellular endocytosis and subcellular
be utilized for functionalization of nanoparticles and targeting
is signiﬁcantly inhibited by MBCD and partially inhibited by sucrose, and sucrose were purchased from Sigma-Aldrich and used as received. Folate-free RPMI-1640 medium and riboflavin free Ham’s F-12K medium were purchased from Invitrogen. Hoechst, and lysotracker red and NBD C6 ceramide green (Golgi tracker green) were purchased from Life Technology.

**Synthesis of β-Cyclodextrin-Functionalized QDs.** Hydrophobic and red emissive CdSe/ZnS-based QDs were synthesized and made hydrophilic by using the previously reported polyacrylate coating method.11,58 First, hydrophobic QDs were dissolved in lgepal−cyclohexane reverse micelles. Next, 0.1 mL N-(3-aminopropyl)-methacrylamide hydrochloride (18 mg dissolved in one mL water), 0.1 mL 3-sulfopropylpropyl methacrylate (228 mg dissolved in one mL water), 0.1 mL poly(ethylene glycol) methacrylate (360 μL dissolved in one mL water), and 100 μL bis[2-(methacryloyloxy)ethyl]phosphate (60 μL dissolved in one mL water) were added. Next, polymerization was initiated under nitrogen atmosphere with the addition of 0.1 mL of persulfate solution (35 mg dissolved in one mL water). After 30 min of polymerization, QDs were precipitated by adding ethanol, and the particles were washed repeatedly with chloroform and ethanol and ﬁnally dissolved in water.

Functionalization with β-CD was performed using NHS-PEG-NHS-based conjugation chemistry. Typically, 100 μL DMF solution of NHS-PEG-NHS (4.6 mg/mL) was mixed with 100 μL borate buffer solution (pH 9.0) of β-CD-NH₂ (11.3 mg/mL). The mixture was shaken for 2–3 min, and then, the whole solution was added to 1–2 mL QDs solution and stirred for 4–5 h at room temperature. Next, the solution was dialyzed against fresh water for 1 day and used as the stock solution.

**Estimation of β-Cyclodextrin in Functionalized QDs.** Detection and quantiﬁcation of QD-bound β-CDs were performed by the anthrone test.59 The anthrone test was performed using different concentrations of β-CD-NH₂. Typically, a stock solution of anthrone was prepared by dissolving 4 mg anthrone in 2 mL of 80% sulfuric acid. Next, 150 μL of β-CD-NH₂ solution was added to it, heated in boiling water bath for 10–15 min, and cooled in ice bath, and the absorbance at 620 nm was measured. A linear calibration curve was obtained by plotting the absorbance with respect to the concentration of β-CD-NH₂. The linear equation was obtained as follows: Y = 2.5 × 10^3X + 0.1 with R² = 0.99. (X = concentration of β-CD-NH₂ and Y = absorbance at 620 nm).

Similarly, the anthrone test was done using CD-functionalized QDs, and the absorbance was measured at 620 nm and the amount of β-CD is determined from the above-mentioned calibration graph. Separately, the concentration of QDs in CD-functionalized QDs was measured by using the QD absorbance at 573 nm.60 Next, the number of CD per QD has been determined from the molar ratio of CD and QDs, and the average value is 70 ± 5. This result was further supported from the GPC-based molecular weight study. The molecular weight

**CONCLUSIONS**

We have shown that supramolecular host−guest chemistry can be utilized for functionalization of nanoparticles and targeting cancer cells followed by cellular endocytosis and subcellular trafficking. In particular, we have synthesized folate and riboflavin-functionalized QDs using CD host and demonstrated their labeling and endocytotic uptake into cancer cells with over-expressed folate/riboflavin receptors. As folate and riboflavin receptors are over-expressed in several types of cancer cells, this approach may be extended for in vivo targeting application via simultaneous delivery of host and guest molecules. In addition, the presented functionalization/labeling approach can be extended to other types of host−guest molecules. Compared to the well-known biotin−avidin interaction, a speciﬁc biological host−guest interaction, this approach is more general with modular interaction and may ﬁnd wider application.
of QD(NH$_2$)$_{100}$ was determined before and after CD conjugation and increased molecular weight of 90 kDa was accounted for ~70 CD. Thus, the CD-functionalized QDs were abbreviated as QD(CD)$_{70}$.

**Preparation of Folate- and Riboflavin-Functionalized QDs via the Host–Guest Interaction.** Aqueous solutions of QD(CD)$_{70}$ (with the CD concentration of 10$^{-4}$ M), DMF solution of folic acid (6 × 10$^{-4}$ M) and aqueous solution of riboflavin (10$^{-4}$ M) were prepared separately. Next, seven sets of folate/riboflavin concentrations, all of them forms an inclusion complex with CD, directs the QD interaction with folate/riboflavin receptors at the cell surface, induces lipid-raft endocytosis of QDs, and traffics QDs toward the perinuclear region. At higher folate/riboflavin concentrations, a significant fraction of them are free for competitive interaction with folate/riboflavin receptors and restrict QD endocytosis.

Figure 7. Subcellular localization of folate-functionalized QDs in KB cells (a) and riboflavin-functionalized QDs in A431 cells (b) and KB cells (c). Colloidal QD(CD)$_{70}$ is incubated with folate, keeping the molar ratio of QD-bound CD to folate at 20 for the preparation of folate-functionalized QDs. Similarly, colloidal QD(CD)$_{70}$ is incubated with riboflavin, keeping the molar ratio of QD-bound CD to riboflavin at 30 for the preparation of riboflavin-functionalized QD. Next, the cells are incubated with the QD sample for 9 h (for folate-functionalized QDs in KB cells) or 3 h (for riboflavin-functionalized QDs) in respective cells followed by incubation with nuclear probes for 30 min, and the washed cells are used for imaging under BF or F mode. Results show that QDs are localized in the perinuclear region and concentrated at one side of the nucleus. Red color corresponds to QDs and blue color corresponds to nuclear probes. Scale bar represents 50 µm.

Scheme 2. Proposed Endocytosis Mechanism of Folate-/Riboflavin-Functionalized QDs Prepared via Supramolecular Host–Guest Chemistry

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activated fetal bovine serum and 1% penicillin streptomycin at overnight in 24-well plates before experiments. KB cells were and A431 cells were used as ribo were used as folate and ribo were used as CD to ribo CD70 and Folic Acid/Ribo were prepared using QD(CD)70 (or ) intensity at 455 nm. Similarly, seven experimental sets were measured (with 370 nm excitation) to note the emission value at 360 and 280 nm, and the emission spectra were followed by neutralizing the solution with NaOH. Other control sets were prepared using β-CD-NH2 instead of using QD(CD)70.

**Study of Host–Guest Complexation between QD-(CD)70 and Folic Acid/Riboflavin.** First, seven experimental sets were prepared using QD(CD)70 (or β-CD-NH2), by varying the CD to folic acid molar ratio from 70 to 1. Next, QD fluorescence was quenched by adding concentrated HCl followed by neutralizing the solution with NaOH. Next, absorption spectra were measured to note the absorbance value at 360 and 280 nm, and the emission spectra were measured (with 370 nm excitation) to note the emission intensity at 455 nm. Similarly, seven experimental sets were prepared using QD(CD)70 (or β-CD-NH2), by varying the CD to riboflavin molar ratio from 70 to 1. Next, QD fluorescence was quenched by adding concentrated HCl followed by neutralizing the solution with NaOH. Next, absorption spectra were measured to note the absorbance of riboflavin at 445 and 370 nm.

**Cell Labeling Study.** Here, in this experiment, KB cells were used as folate and riboflavin receptor over-expressed cells and A431 cells were used as riboflavin receptor over-expressed cells. CHO was used as control cells that does not have over-expressed folate or riboflavin receptors. Cells were cultured overnight in 24-well plates before experiments. KB cells were cultured in folate-free RPMI-1640 (Invitrogen) with 10% heat-activated fetal bovine serum and 1% penicillin streptomycin at 37 °C and 5% CO2 atmosphere. A431 cells were cultured in riboflavin free F-12K (Invitrogen) medium, and CHO cells were cultured in DMEM (Sigma), RPMI, and F-12K (Invitrogen) media with all other conditions similar to the previous one. Next, 50–100 μL QD-folate/QD-riboflavin solution was added followed by 1–9 h incubation. Next, the cells were washed with phosphate-buffered saline (PBS) buffer, and the images were taken by adding fresh medium. The tentative concentration range of QDs, CD, folate, and riboflavin used was 0.1–1.0, 7–70, 0.04–1.4, and 0.04–1.4 μM, respectively.

For the quantitative estimation of cellular uptake, we performed flow cytometry-based study. Cells were separately incubated with QD(NH2)100, QD(CD)70, and folate-/riboflavin-functionalyzed QDs for 3 h and washed with PBS buffer. Then, the cells were treated with trypsin–ethyleneediaminetetra-acetic acid (EDTA) solution to detach them from the plate. Finally, the detached cells were isolated by centrifugation and dispersed in PBS buffer for the flow cytometry study.

**Cell Viability Assay.** KB cells were cultured in 24-well plates in the cell culture medium. After that, cells were treated with the QD sample for 24 h and then washed through PBS buffer, and fresh DMEM medium was added. Then, 50 μL of freshly prepared MTT solution (5 mg MTT in 1 mL deionized water) was added to each well and incubated for 4–5 h. Next, the supernatant was removed and formazan was dissolved in sodium dodecyl sulfate (SDS) solution (8 g of SDS dissolved in 40 mL of DMF–H2O mixture), and the absorbance was measured at 570 nm.

**Instrumentation.** UV–visible absorption spectral studies were carried out with a Shimadzu UV-2550 spectrophotometer. A Malvern Nano ZS instrument was used to measure the DLS size and zeta potential. GPC (Waters S15) equipped with Waters HSP gel columns was used for the determination of the molecular weight. The TEM study was carried out on an FEI Tecnai G2 F20 microscope, fluorescence measurements were performed using a Synergy Mx microplate reader (BioTek) and a PerkinElmer LS 4S, and fluorescence images of cells were captured by using an Olympus IX 81 microscope using Image-Pro Plus 7.0 software. Fluorescence-based quantification was studied using a BD Accuri C6 flow cytometer.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01506.

Details of the endocytosis inhibitor concentration, properties of functional nanoparticles, control cell-labeling experiments, and additional experimental results.

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

The authors acknowledge CSIR, Government of India for financial assistance. (no. 02(0249)/15/EMR-II) S.P. and C.D. acknowledge CSIR, India for providing research fellowship.
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