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In Vitro Evaluation of Dendrimer-Polymer Hybrid Nanoparticles on Their Controlled Cellular Targeting Kinetics

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Abstract

Although polymeric nanoparticles (NPs) and dendrimers represent some of the most promising cancer-targeting nanocarriers, each of them has drawbacks such as limited tissue diffusivity/tumor penetration and rapid *in vivo* elimination, respectively. To address these issues, we have designed a multi-scale hybrid NP system (nanohybrid) that combines folate (FA)-targeted poly(amidoamine) dendrimers and poly(ethylene glycol)-b-poly(D,L-lactide) NPs. The nanohybrids (~100 nm NPs encapsulating ~5 nm targeted dendrimers) were extensively characterized through a series of *in vitro* experiments that validate the design rationale of the system, in an aim to simulate their *in vivo* behaviors. Cellular uptake studies using FA receptor (FR)-overexpressing KB cells (KB FR⁺) revealed that the nanohybrids maintained high FR selectivity resembling the selectivity of free dendrimers, while displaying temporally controlled cellular interactions due to the presence of the polymeric NP shells. The cellular interactions of the nanohybrids were clathrin-dependent (characteristic of polymer NPs) at early incubation time points (4 h), which were partially converted to caveolae-mediated internalization (characteristic of FA-targeted dendrimers) at longer incubation hours (24 h). Simulated penetration assays using multicellular tumor spheroids of KB FR⁺ cells also revealed that the targeted dendrimers penetrated deep into the spheroids upon their release from the nanohybrids, whereas the NP shell did not. Additionally, methotrexate-containing systems showed the selective, controlled cytotoxicity kinetics of the nanohybrids. These results all demonstrate that our nanohybrids successfully integrate the unique characteristics of dendrimers (effective targeting and penetration) and polymeric NPs (controlled release and suitable size for long circulation) in a kinetically controlled manner.

Keywords

Dendrimers; Folic acid; Hybrid nanoparticles; Multi-scale; Targeting

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ASSOCIATED CONTENT

Supporting Information

¹H NMR spectra of G4 PAMAM dendrimer conjugates and PEG-PLA copolymers, UV/Vis spectra of dendrimer-MTX conjugates, SEM images of the nanohybrids, CLSM images of KB FR⁺ and KB FR⁻ cells incubated with the targeted and non-targeted conjugates, z-stack images of MCTS, and cytotoxicity of control conjugates and nanohybrids. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Nanocarriers such as polymer-drug conjugates, dendrimers, polymeric nanoparticles (NPs), micelles, and liposomes have demonstrated great potential to achieve targeted therapy for cancer treatments.^{1–6} Targeting strategies using those nanocarriers include passive targeting based on size control and active targeting via ligand conjugation.^{7–9} To increase the targeting efficacy, integration of the two targeting approaches within a single nanocarrier has been widely attempted using a variety of nanomaterials such as ligand-conjugated polymeric NPs,¹⁰ micelles,¹¹ and liposomes.^{12, 13} However, the single-scale size of these nanocarriers has limitations to optimize their biological properties in terms of biodistribution, tumor targeting, penetration, and cellular uptake, largely because of the different size requirements associated with each of those properties.^{14, 15}

The dense tumor interstitial matrix and abnormal vasculature can lead to inefficient distribution of the drug payloads throughout the tissue.¹⁶ Specifically, the relatively large size of the nanocarriers (50–200 nm) to exploit the enhanced permeability and retention (EPR) effect and targeting moieties exposed on their surfaces can retard tumor penetration due to limited diffusivity and high binding affinity to the superficial tumor cells, respectively.¹⁶ In contrast, smaller NPs (<10 nm) have been shown to achieve enhanced tissue permeation and penetration.^{15–17} In particular, folate (FA)-targeted poly(amidoamine) (PAMAM) dendrimers have previously shown high targeting efficacy to FA receptor (FR)-overexpressing tumor xenografts.^{18–20} However, their small size (~5 nm in diameter) and the surface-exposed targeting ligands have resulted in rapid renal clearance and significant liver uptake, respectively.^{13, 23} Therefore, to maximize the targeting efficacy of drug payloads, a multi-scale nanocarrier, one that combines two or more nanocarriers with different size scales, would be highly desirable. One of the promising ways to achieve the multi-scale system would be to combine actively targeted nanocarriers with favorable tissue penetration and cellular internalization properties, together with larger NPs with a controlled size for passive targeting and long circulation.

Previously, we designed a multi-scale nanocarrier platform by combining linear polymers or targeted dendrimers with larger polymeric NPs.^{21, 22} Generation 4 (G4) PAMAM dendrimers were conjugated with folic acid (FA) as a targeting ligand and encapsulated within poly(ethylene glycol)-b-poly(D,L-lactide) (PEG-PLA) copolymers to produce the hybrid NPs, or nanohybrids, with controlled sizes (~100 nm). The design rationale of the nanohybrid system was to combine the controlled release and larger size of polymeric NPs with the targeting efficacy and favorable tissue penetration of targeted dendrimers. The resulting nanohybrids selectively interacted with FR-overexpressing KB cells (KB FR⁺) in a temporally controlled manner due to the presence of the PEG-PLA shell. Our multi-scale hybrid NPs successfully combined polymeric NPs and targeted dendrimers, allowing precise control over the targeting kinetics by tuning the release profile of the actively targeted dendrimers.²⁷

The dendrimers in the core and polymeric NP shell impart dual properties to the nanohybrid system. This led us to set up a hypothesis that the biological properties of the system may be dictated by one component or the other at a given time, which is dependent upon the dissociation kinetics of the two components. In this paper, we tested this hypothesis by a series of experiments using nanohybrid systems labeled with different fluorophores for the outer shell and dendrimers in the core. First, the effect of incubation time on the selective cellular uptake of the nanohybrids was investigated at various incubation hours (up to 48 h) and compared to free dendrimers and empty polymeric NPs. The cellular association kinetics was also correlated with the release kinetics of the dendrimers in a cell-conditioned culture medium in order to examine the effect of the cellular microenvironment on

dendrimer release. Secondly, the cellular uptake mechanisms of the nanohybrids were investigated by employing metabolic inhibitors, such as methyl- β -cyclodextrin (M β CD) and filipin, which block clathrin- and caveolae-mediated endocytic pathways, respectively. These experiments were designed to elucidate the dominant uptake mechanism(s) of the nanohybrids at various incubation hours. Thirdly, to simulate *in vivo* tumor penetration, multicellular tumor spheroids (MCTS) were used as a 3D *in vitro* model mimicking *in vivo* tumor tissues, allowing evaluation of the penetration ability of the nanohybrids as a function of incubation time. Lastly, the potential of the nanohybrids as a drug carrier was assessed using nanohybrids containing methotrexate (MTX). Our study herein provides fundamental understanding on the kinetically controlled biological properties of the newly developed nanohybrids, which is a key step for further development for *in vivo* applications.

EXPERIMENTAL SECTION

Materials

Generation 4 (G4) PAMAM dendrimer, *N*-hydroxysuccinimide-rhodamine B (NHS-RHO), folic acid (FA), methotrexate (MTX), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), fluorescein isothiocyanate (FITC), filipin, M β CD, glycidol, tin(II)2-ethylhexanoate, poly(ethylene glycol) monomethyl ether (mPEG) (MW 5,000 Da), poly(vinyl alcohol) (PVA, 87–89% hydrolyzed, MW 13,000–23,000 Da), trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and dichloromethane (DCM) were all obtained from Sigma-Aldrich (St. Louis, MO). *D,L*-lactide and Boc-NH-PEG5K-OH were purchased from Polysciences Inc. (Warrington, PA) and Jenkem Technology (Beijing, China), respectively. All other chemicals used in this study were purchased from Sigma-Aldrich unless specified otherwise.

Preparation of G4 PAMAM dendrimer conjugates

Fully hydroxylated RHO-labeled FA-targeted G4 PAMAM dendrimer conjugates (G4-RHO-FA-OH) containing 3.9 RHO and 4.3 FA molecules per dendrimer were prepared and characterized by ^1H NMR as reported in our earlier publication (Figure 1A).^{22–24} MTX was conjugated to the hydroxylated dendrimer conjugates by an ester bond as previously described.²³ Briefly, MTX (1.3 mg, 2.8×10^{-6} mol.) was dissolved in 200 μL DMSO, and EDC (5.4 mg, 2.8×10^{-5} mol.) and NHS (3.2 mg, 2.8×10^{-5} mol.) in 1 mL DMSO were added dropwise under vigorous stirring at RT for 1 h. The activated MTX solution was added dropwise to 10 mg of either G4-RHO-FA-OH or G4-RHO-OH (7.0×10^{-7} mol.) dissolved in 5 mL of ddH₂O, followed by vigorous stirring at RT for 24 h. The solution was then dialyzed in a 3,500 MWCO dialysis membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA) against ddH₂O for 2 days, lyophilized for two days, and stored at -20°C .

Synthesis of FITC-PEG-PLA

PEG-PLA and Boc-NH-PEG-PLA were prepared by ring opening polymerization of *D,L*-lactide as previously described.²² FITC-PEG-PLA was then prepared following deprotection of Boc-NH-PEG-PLA.²⁵ Briefly, Boc-NH-PEG-PLA was deprotected by dissolving 200 mg in 4 mL of DCM, and 4 mL of TFA was added into the solution dropwise under vigorous stirring for 45 min. TFA and DCM were evaporated under vacuum at 70°C using a rotary evaporator. The product was redissolved in 1 mL DCM, precipitated using cold diethyl ether, vacuum filtered, and dried overnight. H₂N-PEG-PLA was conjugated to FITC by dissolving 50 mg (1.2×10^{-3} mmol.) in 2 mL DMF. FITC (0.6 mg, 1.5×10^{-3} mmol.) in 500 μL DMF was added into the polymer/DMF solution under vigorous stirring at RT overnight. Excess FITC was removed by membrane dialysis against ddH₂O using a 3,500 MWCO dialysis membrane for two days. The final product was then lyophilized over 2 days and stored at -20°C .

Encapsulation of the dendrimer conjugates into FITC-labeled PEG-PLA NPs

FITC-labeled hybrid NPs containing targeted or non-targeted dendrimer conjugates (G4-RHO-FA-OH or G4-RHO-OH) were prepared using a double emulsion method.^{21, 22} For example, G4-RHO-FA-OH (100 μ L, 1 mg/mL in ddH₂O) was added to 1 mL of 20 mg/mL solution of PEG-PLA/FITC-PEG-PLA (10:1 w/w) in DCM, and the mixture was sonicated for 1 min using a Misonix XL Ultrasonic Processor (100% duty cycle, 475 W, 1/8" tip, QSonica, LLC, Newtown, CT). Two milliliters of 3% aqueous PVA solution was then added to the mixture, followed by additional sonication for 1 min. The double emulsion was poured into 20 mL of 0.3% PVA in ddH₂O, and vigorously stirred at RT for 24 h to evaporate DCM. The resulting nanohybrid solution was transferred to Nalgene high-speed centrifuge tubes (Fisher Scientific, Pittsburg, PA) to remove PVA and unencapsulated G4-RHO-FA-OH by ultracentrifugation at 20,000 rpm ($48,384 \times g$) for 30 min using a Beckman Avanti J25 Centrifuge (Beckman Coulter, Brea, CA). After washing the nanohybrids five times with ddH₂O, the pellet was resuspended in ddH₂O, lyophilized over 2 days, and stored at -20°C . G4-RHO-OH was also encapsulated into FITC-labeled NPs using the same method. Empty FITC-NPs were prepared by adding 100 μ L ddH₂O instead of the dendrimer solution.

Structure confirmation and size/surface charge measurements

The dendrimer conjugates and PEG-PLA copolymers were characterized by ¹H NMR using a 400 MHz Bruker DPX-400 spectrometer (Bruker BioSpin Corp., Billerica, MA) as described in our earlier publication.²² The MW of PEG-PLA was also measured by Gel Permeation Chromatography (GPC) based on polystyrene standards as previously described.²⁵ Measurements were carried out using a 600 HPLC pump, 717plus Autosampler, and 2414 Refractive Index detector (Waters, Milford, MA, USA) using THF as the mobile phase at 1 mL/min with a Waters Styragel HR2 column at 30°C. The structure of G4-RHO-FA-OH-MTX and G4-RHO-OH-MTX was confirmed by UV/Vis using a DU800 UV/Vis Spectrophotometer (Beckman Coulter, CA). The number of MTX molecules attached to each dendrimer was calculated based on a standard curve of MTX absorbance versus concentration in ddH₂O at 373 nm. Particle size (diameter, nm) and surface charge (zeta potential, mV) of the conjugates and the nanohybrids were measured in triplicates by quasi-elastic laser light scattering using a Nicomp 380 Zeta Potential/Particle Sizer (Particle Sizing Systems, Santa Barbara, CA) in ddH₂O. The measurements were performed using samples that were suspended in ddH₂O at a concentration of 100 μ g/mL, filtered through a 0.45 μ m syringe filter, and briefly vortexed prior to each measurement.

Loading efficiencies of the dendrimer-encapsulated nanohybrids

Loading was defined as the dendrimer conjugate content in the nanohybrids. Five milligrams of each nanohybrid formulation were dissolved in 1 mL of 0.5 M to degrade the PEG-PLA and completely release the loaded dendrimers, followed by filtration through a 0.45 μ m syringe filter. The fluorescence intensity from the filtrates was then measured using a SpectraMAX GeminiXS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA). The amount of the dendrimer conjugates in the filtrates was determined from a standard curve of each conjugate's fluorescence versus concentration in 0.5 M NaOH at 544 nm excitation and 576 nm emission wavelengths. Loading was expressed as μ g dendrimer conjugates per mg copolymer. Loading efficiency was defined as the ratio of the actual loading obtained to the theoretical loading.

Scanning Electron Microscopy (SEM) observations

Surface morphology of the nanohybrids was examined by scanning electron microscopy (SEM) using a JEOL-JSM 6320F field emission microscope (JEOL USA, Peabody, MA) as

previously described.^{21, 22} Samples were sputter-coated with Pt/Pd at a coating thickness of 6 nm (Polaron E5100 sputter coater system, Polaron, UK) and then visualized at an accelerating voltage of 4.0 mV and 8.0 mm working distance.

Cell culture

The KB cell line was purchased from the American Type Tissue Collection (ATCC, Manassas, VA) and grown continuously as a monolayer at 37 °C, 5% CO₂ in GIBCO RPMI 1640 medium (Invitrogen Corporation, Carlsbad, CA), resulting in FR-downregulated KB cells (KB FR⁻). The RPMI 1640 medium was supplemented with penicillin (100 units/mL), streptomycin (100 mg/mL), and 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) before use. Some of the cells were cultured in FA-deficient RPMI 1640 media (Invitrogen) for at least 4 days before experiments, resulting in FR-overexpressing KB (KB FR⁺) cells.^{22, 26}

Dendrimer release kinetics in cell-conditioned culture media

KB FR⁺ cells were seeded in 12-well plates at a density of 2×10^5 cells/well in complete FA-deficient RPMI 1640 for 24 h. The complete medium was then replaced with basal FA-deficient RPMI 1640, and the cells were incubated up to 48 h. At 1, 4, 24, and 48 h, the media were withdrawn and used to dissolve the nanohybrids in triplicate to achieve a concentration equivalent to 100 nM G4-RHO-FA-OH. The nanohybrids were then incubated for 1, 4, 24, and 48 h. At the end of each incubation time, the solutions were centrifuged at 14,000 rpm ($23,708 \times g$) for 5 min to precipitate intact nanohybrids, and the fluorescence intensities of the supernatants were measured. The amounts of the dendrimer conjugates released over time were determined based on a standard curve of G4-RHO-FA-OH fluorescence versus concentration in basal FA-deficient RPMI 1640, as described above.

Cellular interactions of the nanohybrids labeled with two dyes (RHO and FITC)

KB FR⁺ cells were seeded in 4-well chamber slides (Millicell EZ Slide, Millipore, Billerica, MA) at a density of 1.0×10^5 cells/well and incubated in FA-deficient RPMI 1640 for 24 h. The cells were then treated with G4-RHO-FA-OH, G4-RHO-OH, the corresponding FITC-labeled nanohybrids, and empty FITC-NPs for 30 min (G4-RHO-FA-OH only), 1, 4, 24, and 48 h, at a concentration of 100 nM based on the dendrimer conjugates in basal FA-deficient RPMI 1640. Additionally, KB FR⁻ cells were also used as a negative control and incubated with G4-RHO-FA-OH and its nanohybrid formulation. After each incubation time, cells were washed twice with PBS with Ca⁺⁺/Mg⁺⁺ (Mediatech, Inc., Manassas, VA), fixed in 4% paraformaldehyde for 10 min, and washed again. The slides were then mounted with antiphotobleaching mounting media with DAPI and covered with glass coverslips for confocal observations.

Cellular interactions of the nanohybrids in the presence of endocytic inhibitors

KB FR⁺ cells were seeded in 4-well chamber slides as described above, and then treated with filipin (1 µg/mL),²⁷ MβCD (5 mM),²⁸ or a mixture of filipin and MβCD in FA-deficient basal RPMI 1640 for 1 h. Cells were washed once with PBS with Ca⁺⁺/Mg⁺⁺ followed by adding G4-RHO-FA-OH (100 nM), the corresponding nanohybrids, and empty FITC-NPs. The treatment was carried out for 4 and 24 h, after which the confocal samples were prepared as described above.

Penetration assay using multicellular tumor spheroids (MCTS)

MCTS formation was performed using the liquid overlay method.²⁹ KB FR⁺ cells from a confluent T-75 flask were detached using trypsin-EDTA and resuspended in FA-deficient RPMI 1640 at a concentration of 6×10^3 cells/mL. Five hundred microliters of the cell

suspension were transferred to 8-well chamber slides (Millicell EZ Slide, Millipore, Billerica, MA) coated with 1% agarose in complete FA-deficient RPMI 1640. The cells were then incubated on agarose for 7 days to allow the formation of MCTS. After 7 days, 250 μ L of the media in each well were removed, and MCTS were treated with 250 μ L of 200 nM G4-RHO-FA-OH, an equivalent concentration of the nanohybrids, or empty FITC-NPs, for 1, 4, 24, and 48 h. After each treatment, MCTS were carefully washed twice with PBS with $\text{Ca}^{++}/\text{Mg}^{++}$ fixed in paraformaldehyde for 10 min, and washed again. The chamber gasket was then removed, and the pieces of agarose were transferred to glass cover slips for confocal observation.

Confocal microscopy observation

Cells incubated with various nanomaterials as described above were visualized using a Zeiss LSM 510 Meta confocal laser scanning microscope (CLSM, Carl Zeiss, Germany). A 25 mW diode UV 405 nm laser was used for excitation of DAPI, the 543 nm line of a 1 mW tunable HeNe laser was used for excitation of RHO, and the 488 nm line of a 30 mW tunable Ar laser was used for the excitation of FITC. Emission was filtered at 420 nm, 565–595 nm, and 505–530 nm for DAPI, RHO and FITC, respectively. Images were captured using a 63x/1.2 Water DIC C-Apochromat objective. Z-stack images for the 2D cell culture experiments were taken at 1 μ m intervals for a total slice thickness of 8 μ m. For the penetration assay, MCTS were viewed using a 10x/0.25 Ph1 A-Plan objective, and Z-stack images were taken at 20 μ m intervals for a total slice thickness of 140 μ m.

Cytotoxicity assay of the MTX-conjugated dendrimers and nanohybrids

Each of G4-RHO-FA-OH-MTX, G4-RHO-OH-MTX, or free MTX was encapsulated into PEG-PLA NPs using the double emulsion method as described above. KB FR⁺ cells were seeded in 96-well plates at a density of 5×10^3 cells/well for 24 h. The cells ($n = 4$) were then treated with 1 μ M free MTX or an equivalent concentration of the dendrimer conjugates, nanohybrids, or MTX-encapsulated NPs in basal FA-deficient RPMI 1640 for 1, 4, 24, 48, and 72 h. After each incubation time, the media was replaced with complete FA-deficient RPMI 1640 and the cells were further incubated for 72 h to allow them to proliferate, while changing the media after 48 h. At 72 h post-treatment, the media was replaced, and the MTS assay reagent (CellTiter 96 AQueous One Solution (MTS) Assay, Promega, Madison, WI) was added to each well. The cells were incubated for 2 h, and the plates were read at 492 nm absorbance wavelength. Cell viability was expressed as % proliferation relative to untreated cells and plotted against incubation time. A similar experiment was conducted with the control conjugates without MTX (G4-RHO-FA-OH and G4-RHO-OH and their nanohybrids) and empty NPs.

RESULTS AND DISCUSSION

Preparation of the G4 PAMAM dendrimer conjugates and nanohybrids

A general overview of the preparation of the nanohybrids is illustrated in Figure 1. RHO-labeled, FA-targeted G4 PAMAM dendrimers were prepared by sequential conjugation with RHO and FA, followed by hydroxylation of the remaining amine groups, resulting in G4-RHO-FA-OH (Figure 1(A)). The hydroxylation step was performed to eliminate non-specific interactions between amine-terminated dendrimers and cell membranes as well as to enable subsequent conjugation with MTX through an ester bond.²³ Conjugation of RHO and FA to the dendrimers and successful end-capping of the amine groups was confirmed using ¹H NMR and zeta potential measurements (Table 1 and Figure S1). The ¹H NMR spectra (Supporting Information Figure S1) revealed that the conjugates prepared in this study contained approximately 3.9 and 4.3 RHO and FA molecules per dendrimer, respectively. Since the characteristic proton peaks of MTX were overlapping with those of

FA and RHO, the number of MTX molecules per dendrimer could not be estimated by ^1H NMR. For this reason, MTX conjugation was confirmed using UV/Vis (Supporting Information Figure S2), which revealed that there are approximately 4.7 and 5.6 MTX molecules attached to G4-RHO-FA-OH and G4-RHO-OH, respectively.

PEG-PLA and FITC-PEG-PLA copolymers were synthesized by bulk polymerization of *D,L*-lactide using mPEG5K and Boc-NH-PEG5K-OH as initiators. ^1H NMR was used to confirm the chemical structure of the copolymers and to estimate the MW of the PLA block (Supporting Information Figure S3). This was calculated to be 44,900 g/mol for PEG-PLA (M_n : 37,500, M_w : 45,521, PDI: 1.21 as measured by GPC) and 48,800 g/mol for Boc-NH-PEG-PLA based on the relative integration ratios of peak b around 3.62 ppm (the protons of the ethylene oxide repeating units) to peak c around 5.15 ppm (the lactide repeating units). Following deprotection of Boc-NH-PEG-PLA, H_2N -PEG-PLA was obtained and conjugated to FITC, which was also confirmed using ^1H NMR (Supporting Information Figure S3).

The various dendrimer conjugates were then encapsulated into PEG-PLA copolymers using the double emulsion method to produce the nanohybrids as we described earlier (Figure 1(B)).²² Dendrimer encapsulation was performed using double emulsion to prepare nanohybrids with controlled particle sizes around 100 nm in diameter (Supporting Information Figure S4) and at high loading efficiencies (67 – 83%) (Table 1). The change in zeta potential values for the nanohybrids (–11.6 – –18.6 mV) compared to those of the dendrimer conjugates before encapsulation (3.4 – 28.1 mV) indicated successful encapsulation.

Selective cellular interactions of the nanohybrids containing targeted dendrimers

Non-targeted polymeric NPs have been reported to exhibit a degree of non-specificity when incubated with cells.^{21, 30} Targeted dendrimers on the other hand have shown excellent receptor selectivity with minimal non-specific uptake after neutralization of the surface groups.^{22, 26} Our design strategy is based on hybridization of the two nanocarriers, which is hypothesized to impart both dendrimer- and NP-like attributes to the nanohybrid system. To test this hypothesis, we first investigated whether the targeting efficiency of the system is more affected by the dendrimers (highly selective) or by the polymeric NPs (non-specific). We previously monitored the cellular uptake of the FA-targeted nanohybrids based on RHO conjugated to the dendrimers, showing high selectivity of the nanohybrids to KB FR⁺ cells up to 4 h of incubation.²² In this paper, we conducted a similar experiment using a two-dye system, i.e., FITC-labeled NPs encapsulating RHO-labeled dendrimers, and observed the cellular interactions of the nanohybrids up to 48 h of incubation. As similarly observed in our previous report, after 1 h of incubation, only the free targeted dendrimers (G4-RHO-FA-OH) showed significant cellular binding and uptake into KB FR⁺ (Supporting Information Figure S5). The targeted nanohybrids (G4-RHO-FA-OH/FITC-NP) started to selectively interact with the cells after 4 h, likely due to the protective effect of the polymeric shell (Figure 2 and Supporting Information Figure S5). The overlap in the red and green fluorescence signals in those images shows the co-localization of the dendrimers with the nanohybrid shell, indicating that the nanohybrids interacted with cells intact. This interaction was seemingly specific, as the fluorescence from the targeted nanohybrids was significantly higher than that of the non-targeted nanohybrids (G4-RHO-OH/FITC-NP) and that from KB FR[–] cells (Supporting Information Figure S6). Green fluorescence signals from empty FITC-NPs were also negligible (Figure 2 and Supporting Information Figure S5). Our observations indicate that the targeted dendrimers, particularly those near the surface of the nanohybrids, may act as a driving force that contributes to the selective association with FR on the cells. After 24 h, the targeted nanohybrids still showed a degree of overlap in the red and green signals; however, an increase in the red fluorescence intensity relative to the green signal was observed from the cells when compared to that at 4 h. The separation of the red

signal from the green fluorescence is a clear indication of the dendrimer release from the nanohybrids after 24 h of incubation, which became predominantly observed from the cells at the longer incubation hours (24–48 h). After 48 h of incubation, non-specific cellular interactions were observed as non-targeted systems and the empty FITC-NPs started to interact with the cells (Supporting Information Figure S5 and Figure S6).

The cellular microenvironment facilitates dendrimer release from the nanohybrids

Release kinetics of macromolecules from polymeric NPs with similar MW to the PEG-PLA copolymers used in this study are typically slow and can take up to several days to weeks to complete the release. For example, we have previously conducted a release study of the dendrimers from the nanohybrids in PBS and found that only 18% and 38% of the dendrimer conjugates are released after 4 and 24 h, respectively.²² Unexpectedly, Figure 2 shows that the cellular uptake kinetics was much faster and completed within 48 h, which is likely due to the presence of cells that accelerate the release kinetics. We thus investigated the effect of the cellular microenvironment on the dissociation and release of the dendrimer conjugates from the nanohybrids. The release medium used for this experiment was the same culture medium used for the cellular uptake studies after conditioning with cells for various incubation hours. As shown in Figure 3, the release kinetics in conditioned culture medium was significantly faster (38% and 60% release after 4 and 24 h, respectively) than that obtained using PBS. This accelerated release profile confirmed that the selective cellular interaction observed in Figure 2 is primarily a result of the release of the targeted dendrimers from the nanohybrids particularly at longer incubation hours. By conducting the release test in the conditioned media, we can better understand the effect of the cellular microenvironment on the release kinetics of the nanohybrids. This therefore serves as a valuable *in vitro* tool that can help predict the *in vivo* behavior of the nanohybrids.

Dual properties in cellular interactions of the nanohybrids revealed by endocytic inhibitors

Nanocarriers (both targeted and non-targeted) are known to be associated with various internalization mechanisms such as endocytosis (clathrin-, caveolae-mediated, or non-specific adsorptive endocytosis), energy-independent cell entry, and macropinocytosis.^{30–32} Although controversial, polymeric NPs have been reported to internalize into cells through non-specific pathways that are frequently associated with clathrin-mediated endocytosis, whereas FA-targeted dendrimers reportedly utilize caveolae-mediated endocytosis similar to other FA-targeted systems.³³ M β CD is a commonly used agent for cellular uptake studies and is known to extract cholesterol from membranes, which inhibits clathrin-coated pit formation and subsequent endocytosis.²⁸ Filipin on the other hand is known to inhibit caveolae-mediated endocytosis.²⁷ We compared the cellular uptake mechanism of the FA-targeted nanohybrids to that of free FA-targeted dendrimers and non-targeted empty FITC-NPs under the presence of filipin, M β CD, or a combination of the two agents, at various incubation times. As shown in Figure 4 and Supporting Information Figure S7, cellular uptake of G4-RHO-FA-OH was inhibited by filipin and filipin/M β CD, but not affected by M β CD alone up to 24 h. In contrast, the internalization of the nanohybrids was inhibited by both M β CD and filipin/M β CD at 4 h but only by filipin/M β CD after 24 h. These observations indicate that the cellular interaction of the nanohybrids follows a similar pathway to polymeric NPs at early incubation times. After 24 h, as more dendrimers are released, the nanohybrids exhibit characteristics of both dendrimers and NPs, requiring the blockade of the two pathways to inhibit their internalization. The cellular uptake inhibition of empty FITC-NPs at 4 h (Supporting Information Figure S7) was not as obvious since the incubation time may not have been enough to achieve significant non-specific internalization of the NPs. However, inhibition was observed at 24 h by M β CD and filipin/M β CD, confirming that the cellular uptake of NPs was dependent on non-specific clathrin-mediated mechanism. Even though the inhibitory effect of filipin and M β CD was partially

reversed by 24 h resulting in incomplete inhibition for some groups (e.g. G4-RHO-FA-OH at 24 h, Supporting Information Figure S7), these results confirm that the nanohybrids possess both NP-like and dendrimer-like characteristics in an incubation time-dependent manner.

Targeted dendrimers penetrate tumor spheroids following their release from the nanohybrids

Tumor spheroids generated by the liquid overlay method can serve as a reliable *in vitro* 3D tumor model. Their multicellular organization represents not only cell aggregates, but has also been reported to contain an organized extracellular matrix resembling that of tumors *in vivo*.^{34, 35} The small size, molecular flexibility, and deformability of dendrimers have been shown to contribute to their highly efficient tissue penetration through tumors and 3D tumor models such as MCTS.^{36, 37} Using MCTS, we assessed tumor penetration of the FA-targeted dendrimers and nanohybrids up to 48 h incubation. Figure 5 shows confocal images taken at a depth of 80 μ m into each spheroid (individual z-stack images for each group are shown in Supporting Information Figure S8). Free G4-RHO-FA-OH starts to penetrate into the spheroids within 1 h. For the nanohybrids, a delay up to 4 h in spheroid penetration was observed, followed by substantially increased red signals, representing that the released dendrimers have reached the core of the spheroids. The absence of green signals in the core of the spheroids treated with the nanohybrids strongly indicates that only the released dendrimers were able to penetrate as deep as free dendrimers, while the intact nanohybrids remained at the periphery. A similar observation, with more nanohybrids clustered at the MCTS periphery, was obtained at 24 and 48 h. As expected, empty FITC-NPs were not able to penetrate the spheroids, remaining on top or at the periphery. The results highlighted in Figure 5 and Supporting Information Figure S8 serve as *in vitro* validation for the design rationale of our multi-scale nanohybrid system, where efficient tumor penetration can be achieved by the smaller, highly flexible dendrimers upon release from the NP shell that has suitable size for passive targeting to tumors.

The nanohybrid platform enables temporal control over cytotoxicity

In order to validate the drug delivery potential of the nanohybrids, we employed MTX as a model chemotherapeutic drug.^{18, 19} For this experiment, MTX was conjugated to the targeted and non-targeted dendrimer conjugates, followed by encapsulation into PEG-PLA, resulting in the MTX-containing nanohybrids. As shown in Figure 6, no significant inhibition in cell proliferation was observed within the first 4 h of incubation, which can be attributed to the incomplete release of the drug and drug conjugates from the various systems. After 24 h, free MTX and the targeted dendrimer-MTX conjugates (G4-RHO-FA-OH-MTX) showed a significant inhibition in cell proliferation, and a less but obvious anti-proliferation effect was observed from the group treated with the targeted nanohybrids (G4-RHO-FA-OH-MTX/NP). The targeted nanohybrids showed a delay in the cellular uptake of the drug conjugates, which translated into a delay in the cytotoxic effect of MTX, while maintaining the similar receptor selectivity as the free targeted dendrimers. After 72 h of incubation, the targeted nanohybrids maintained their selectivity as shown in the significantly higher cytotoxic effect than the non-targeted nanohybrids containing G4-RHO-OH-MTX and MTX-encapsulated NPs. The time delay in the release of the targeted dendrimer-drug conjugates from the nanohybrids warrants the long circulation of the systems, while allowing the nanohybrids to exhibit free dendrimer-like behavior at a certain period of time after injection. These results provide a promising starting point for future *in vivo* translation of this system.

CONCLUSIONS

Taken together, the results highlighted herein demonstrate the temporally controlled, dual nature of the multi-scale nanohybrid platform. Through a series of *in vitro* experiments that simulate *in vivo* situations, our results indicate that the dendrimer-polymer nanohybrid system combines the characteristics of free dendrimers, such as high receptor selectivity and efficient tumor penetration, with the controlled release properties and larger size of the polymeric NPs. Co-localization of the dendrimers and the polymeric NP shell of the nanohybrids showed the selective uptake of the intact nanohybrids at 4 h of incubation (Figure 2). The release of free dendrimers, which was facilitated in the culture medium (Figure 3), enhanced the selective uptake of the nanohybrids up to 24 h, as supported by the increased red fluorescence signals from KB FR⁺ observed at longer incubation times (Figure 2). Inhibition of different endocytic pathways revealed that the uptake mechanism of the nanohybrids closely resembles polymeric NPs at earlier time points, and follows both dendrimers- and NP-like pathways at longer incubation times (Figure 4). MCTS served as an effective 3D *in vitro* model for tumor penetration and showed the released dendrimers from the nanohybrids can still achieve efficient penetration similar to free dendrimers (Figure 5). Finally, the *in vitro* cytotoxicity results (Figure 6) demonstrate the high FR selectivity of the FA-targeted dendrimer-MTX conjugates and their nanohybrids. In addition to the selective toxicity, the targeted nanohybrids also exhibited the controlled cellular uptake kinetics that is attributed to the nanohybrid design. These results will be further validated *in vivo* to demonstrate the tumor targeting efficacy of the nanohybrids through a combination of longer circulation time and enhanced tumor selectivity and penetration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Overview of nanohybrid preparation. (A) Sequential preparation of the targeted dendrimer conjugates, (B) Encapsulation of the dendrimer conjugates into PEG-PLA copolymers to produce the nanohybrids.

Figure 2.

Ortho view of Z-stack images of KB FR⁺ cells upon incubation with G4-RHO-FA-OH (left column), G4-RHO-FA-OH-encapsulated nanohybrids (middle column), and empty FITC-NPs (right column) at 4 and 24 h (the complete set of CLSM images up to 48 h is shown in Supporting Information Figure S5). Red: RHO-labeled dendrimers, green: FITC-labeled NPs, blue: cell nuclei stained by DAPI, scale bar: 10 μ m. The targeted dendrimers show specific interaction with KB FR⁺ throughout the incubation period. The targeted nanohybrids start to selectively interact with the cells after 4 h as the overlapping red and green fluorescence signals are observed. At longer incubation hours (24 h and Supporting Information Figure S5), the red signals become predominant, indicating that the released dendrimers selectively interact with the KB FR⁺ cells. The empty NPs start to interact with the cells after 24 h likely due to non-specific interactions. Note that non-targeted dendrimers and nanohybrids show significantly less cellular interaction up to 48 h (Supporting Information Figure S6).

Figure 3.

Release kinetics of G4-RHO-FA-OH/FITC-NP in cell-conditioned basal FA-deficient RPMI 1640 media. Faster release kinetics were obtained compared to the release profile in PBS (red dotted line, adapted from ref. 22), with ~90% of the dendrimer conjugates released after 48 h of incubation.

Figure 4.

Effect of endocytic inhibitors on cellular interactions of the nanohybrids observed using CLSM. KB FR⁺ cells were incubated with G4-RHO-FA-OH, nanohybrids (G4-RHO-FA-OH/FITC-NP), and empty FITC-NPs for 4 h and 24 h (the complete set of images is shown in Supporting Information Figure S7). Red: RHO-labeled dendrimers, green: FITC-labeled NPs, blue: cell nuclei stained by DAPI, scale bar: 20 μ m. Cellular uptake of the targeted dendrimers is fully inhibited at 4 h by filipin and filipin/M β CD, but not affected by M β CD alone. The uptake of the targeted nanohybrids is inhibited by M β CD and filipin/M β CD at 4 h, exhibiting polymeric NP-like behavior. However, at 24 h, only filipin/M β CD blocks the interaction of the nanohybrids, and yet limited effect of M β CD is observed, indicating the selective cellular interactions by the released dendrimers (red fluorescence). As expected, non-specific uptake of the empty FITC-NPs at 24 h is inhibited by M β CD and filipin/M β CD.

Figure 5.

(A) CLSM images of KB FR⁺ MCTS upon incubation with G4-RHO-FA-OH, G4-RHO-FA-OH/FITC-NP, and empty FITC-NPs up to 48 h. Red: RHO-labeled dendrimers, green: FITC-labeled NPs. Images shown were taken at a depth of 80 μ m into each spheroid, scale bar: 100 μ m. (B) Fluorescence intensities from the core of the MCTS quantified by ImageJ. Only the free dendrimers and those released from the nanohybrids are able to penetrate deep into the spheroids. Empty FITC-NPs and intact nanohybrids accumulate at the periphery of the spheroids even after 48 h.

Figure 6.

Cell proliferation kinetics of KB FR⁺ (n = 4) after treatment with MTX-conjugated dendrimers and nanohybrids at a concentration equivalent to 1 μ M MTX over 72 h. The MTS assay was performed after additional 72 h to allow the cells to proliferate. Free MTX and the MTX-conjugated FA-targeted dendrimers start to exhibit cytotoxicity after 24 h, as indicated by the reduction in cell proliferation (<80%) relative to untreated controls. G4-RHO-FA-OH-MTX-encapsulated nanohybrids show a similar effect on cell growth at the 72 h time point but with a time delay of 48 h due to the controlled release of the dendrimer conjugates. * and ** denote significant difference in cell proliferation relative to the non-targeted dendrimer conjugates (G4-RHO-OH-MTX) and nanohybrids (G4-RHO-OH-MTX/NP), respectively. Statistical analysis was performed using OriginPro 8.5 using 1-way ANOVA followed by Tukey's post hoc test at $p < 0.05$.

Table 1

Characterization of the various G4 PAMAM dendrimers and nanohybrids

	Particle size (nm)	Zeta potential (mV) [*]	Loading efficiency (EE, %) ^{***}
G4-RHO-NH ₂	16.7 ± 2.4	28.1 ± 1.8	N/A
G4-RHO-OH	12.1 ± 7.3	4.2 ± 1.7	N/A
G4-RHO-OH-MTX	15.2 ± 3.2	2.5 ± 0.7	N/A
G4-RHO-FA-OH	19.6 ± 7.8	3.4 ± 1.6	N/A
G4-RHO-FA-OH-MTX	13.4 ± 5.3	3.2 ± 1.1	N/A
FITC-NP	114.7 ± 5.7	-13.5 ± 4.2	N/A
G4-RHO-OH/FITC-NP	101.5 ± 8.5	-11.6 ± 3.3	69.0
G4-RHO-OH-MTX/NP	89.8 ± 12.5	-18.6 ± 3.5	83.1
G4-RHO-FA-OH/FITC-NP	125.5 ± 10.2	-15.3 ± 6.5	72.5
G4-RHO-FA-OH-MTX/NP	79.8 ± 5.4	-14.4 ± 5.7	67.2

^{*} Measured in ddH₂O, pH 5.6

$$^{**}EE(\%) = \frac{\text{Encapsulated mass of the dendrimers}}{\text{Originally added mass of the dendrimers}} \times 100$$