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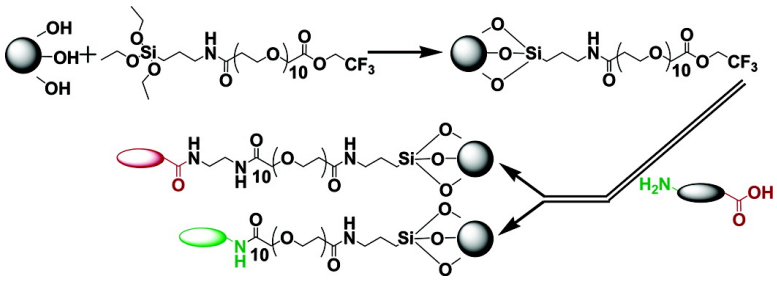

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A Bifunctional Poly(ethylene glycol) Silane Immobilized on Metallic Oxide-Based Nanoparticles for Conjugation with Cell Targeting Agents

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Abstract: A trifluoroethylester-terminal poly(ethylene glycol) (PEG) silane was synthesized and self-assembled on iron oxide nanoparticles. The nanoparticle system thus prepared has the flexibility to conjugate with cell targeting agents via either carboxylic or amine terminal groups for a number of biomedical applications, including magnetic resonance imaging (MRI) and controlled drug delivery. The trifluoroethylester silane was synthesized by modifying a PEG diacid to form the corresponding bistrifluoroethylester (TFEE), followed by a reaction with 3-aminopropyltriethoxysilane (APS). The APS coupled with PEG chains confers the stability of PEG self-assembled monolayers (SAMs) and increases the PEG packing density on nanoparticles by establishing hydrogen bonding between the carbonyl and amine groups present within the monolayer structure. The success of the synthesis of the PEG TEFE silane was confirmed with ^{1}H NMR and Fourier transform infrared spectroscopy (FTIR). The conjugating flexibility of the PEG TEFE was demonstrated with folic acid that had carboxylic acid groups and amine terminal groups, respectively, and was confirmed by FTIR. TEM analysis showed the well-dispersed nanoparticles before and after they were coated with PEG and folic acid.

Introduction

Magnetic resonance imaging (MRI) is an appealing noninvasive approach for early cancer diagnostics and therapeutics.[1] While the imaging capabilities of these instruments have revolutionized imaging technology, the resolution of the instrument is limited to the elucidations of lesions within the body on the order of 1 mm. This limitation of the instrument has led to the development of several types of contrast enhancement agents including magnetite/dextran-based nanoparticles and chelated gadolinium contrast agents, which are currently available on the market and used widely in clinical applications. However, Gd complex contrast agents are effective only when present in millimolar concentrations.[1] Because of the superparamagnetic property, iron oxide nanoparticles have been found effective in nanomolar concentrations[1] and can better serve as contrast enhancement agents for MRI.[2,3] Apart from serving as contrast enhancement agents, superparamagnetic nanoparticles can be used as drug carriers for controlled drug release; they can accumulate in tumors very efficiently through enhanced permeation and retention.[4] It is envisioned that nanoparticles can be surface-modified to function as both contrast enhancement agents and drug carriers simultaneously, allowing real-time monitoring of tumor response to drug treatment.

However, when nanoparticles are conjugated with target agents or undergo any surface modification, particle agglomeration as a result of their large surface-to-volume ratio becomes a primary concern. When nanoparticles agglomerate, they not only lose their intended functionality, but can also be quickly cleared by macrophages or accumulated in the reticule-endothelial system before they can reach the target cells.[5] One approach to solving this problem is to modify the particulate surface with poly(ethylene glycol) (PEG) self-assembled monolayers.[6] Surfaces covered with PEG have proven to be nonimmunogenic, nonantigenic, and protein resistant.[7,8] While the PEG moiety provides an efficient system to increase particulate circulation time in blood, the nanoparticle systems must also be coupled with tumor targeting agents to be useful for the intended applications. Thus, the PEG moiety immobilized on nanoparticles must also provide an active functional group capable of conjugating with targeting agents.

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PEG is used widely to functionalize proteins and peptides for drug delivery.[9,10] Research in cell targeting has also utilized functional PEG molecules conjugated with folic acid on liposomes.[11] Monofunctional PEG molecules coupled to proteins are known to prolong the particle circulation time in blood and reduce immunogenicity.[12−14] While functionalized carboxyl or amine PEGs are widely available, they remain expensive and require chemical modification to convert to their corresponding silanes. Further, these functional PEGs are available mainly in high molecular weights, which may inhibit PEG monolayer self-assembly on the nanoparticle surface due to the labile nature of PEG molecules. Currently available PEGs can conjugate with only one type of functional group present in targeting agents, typically either amine or carboxyl groups, and, in most of cases, they are not suitable for nanoscaled devices such as nanoparticle systems due to their high molecule weight. PEG silylation normally occur in organic solution, whereas conjugation with tumor targeting agents such as folic acid or antibodies needs to be conducted in aqueous solution. Thus, PEG self-assembled monolayers (SAM) must be flexible enough to prevent agglomeration during solvent exchange and remain active in solvents to provide a terminus for conjugation.

In this research, we have developed a novel, inexpensive bifunctional trifluoroethylester (TFEE) PEG silane that has low molecular weight and can easily self-assemble on metal oxide-based nanoparticles. This nanoparticle-PEG silane system has the following advantages: (1) The TFEE PEG has low molecular weight and is especially suitable for cell targeting where the nanoscale dimensions must be enforced. (2) The TFEE PEG silane is capable of conjugating with both amine and carboxylic groups, the two types of functional groups present in many targeting agents for cancer treatment. The TFEE terminal group provides an easily manipulated headgroup capable of amidation via primary amines in organic solutions or that is hydrolyzed following self-assembly in aqueous conditions as demonstrated by Fryxell and colleagues.[15] The formation of the TFEE end group provides a stable leaving group capable of undergoing smooth amidation through the addition of a primary amine. (3) The 3-aminopropyltriethoxysilane (APS) coupled with the PEG chains confers the stability of PEG self-assembled monolayers (SAMs) and increases PEG packing density on nanoparticles by establishing hydrogen bonding between PEG interchains.[16] (4) The nanoparticles modified with TFEE silane are well dispersed, which is an essential requirement for in vivo applications to ensure the nanoparticles have the desired targeting functionality, biocompatibility, and long blood circulation time.

To demonstrate the effectiveness of ligand immobilization, folic acid (FA), a widely used targeting agent containing primary amines and carboxylic acid groups native to the structure, was chosen as our model system. Folic acid is a low molecular targeting agent whose corresponding receptor is overexpressed on many types of cancer cells. The structure of FA allows conjugation through the TFEE group of the PEG terminus and primary amine on the FA, or the TFEE terminal group can be converted to a primary amine through the addition of ethylenediamine (EDA) which can then be reacted with the carboxyl groups present on the FA. The short chain of folic acid molecules also allows the efficient internalization of nanoparticles by target cells, in contrast to the widely used antibodies which are bulky and thus have difficulty crossing the cell membrane.[6,17]

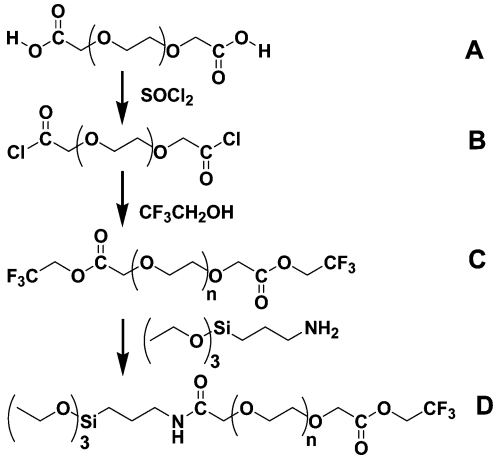

Figure 1. Chemical scheme for the synthesis of a trifluoroethylester-terminal PEG silane.

2. Experimental Section

Materials. The following materials were purchased from Sigma-Aldrich and used as received: PEG biscarboxylate (M_n 600), thionyl chloride, trifluoroethanol, ethylenediamine, 3-aminopropyltriethoxysilane (APS), *N*-hydroxysuccinimide 97% (NHS), 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide (EDAC), iron(III) acetylacetonate, diphenyl ether, oleylamine, oleic acid, folic acid, and tertiarybutoxycarbonyl. Benzene-d_6 was purchased from Cambridge Isotopes. All other solvents were purchased from Fisher Scientific (Hampton, NH) or Aldrich (Milwaukee, WI).

Synthesis of PEG Silane. The synthetic process for the PEG-trifluoroethylester was modified from previous alkane SAM synthetic methods.[15,18] The process and products for each reaction step are shown schematically in Figure 1. In a round-bottom flask, 100 g (0.167 mol) of PEG biscarboxylate was degassed under a 2-Torr vacuum to remove residual water and air in the liquid. Following degassing, 35 mL (0.48 mol) of thionyl chloride was added dropwise to the neat PEG, converting it to the corresponding diacid chloride. Initially, vigorous bubbling was observed followed by slow bubbling resulted from HCl and SO_2 gas. The sample was heated for 1.5 h under nitrogen followed by degassing under a 2-Torr vacuum to remove SO_2 gas and excess thionyl chloride. The crude diacid chloride displayed the following ^{1}H NMR spectrum: (C_6D_6) δ 4.08 (brm, 4H, ClOCCH_2−), 3.4 (brm, 43H, −CH_2CH_2O−).

Next, 32 mL (0.44 mol) of 2,2,2-trifluoroethanol was added dropwise to the PEG diacid chloride under nitrogen. The solution was stirred for 2 h and heated to reflux for 3 h. After being cooled to room tem-

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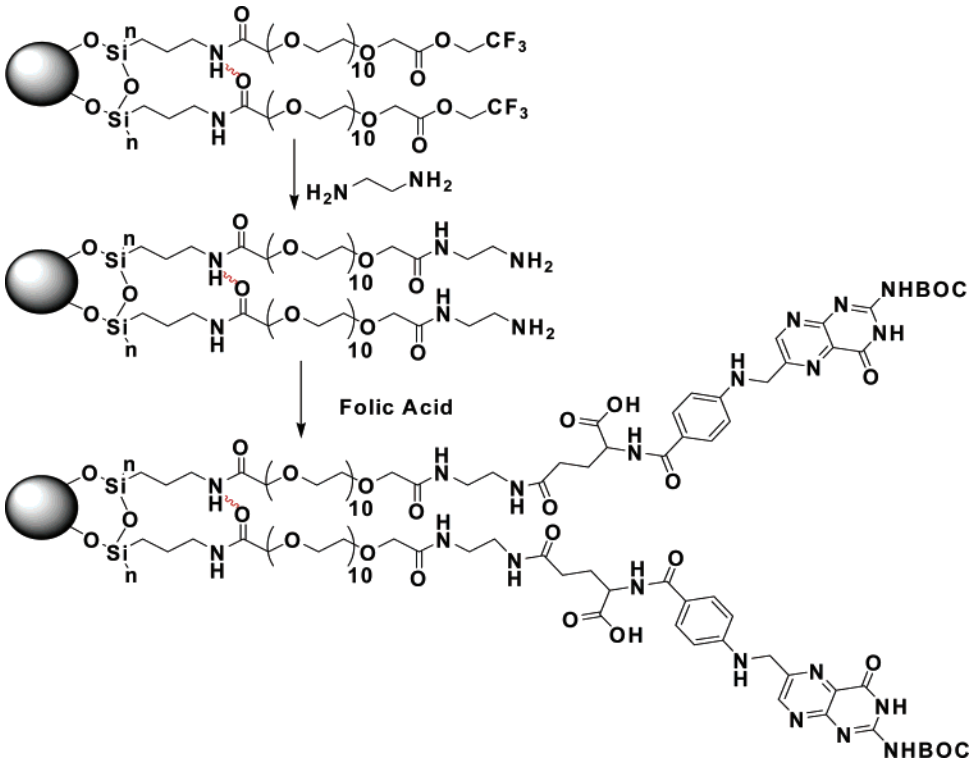

Figure 2. Immobilization of PEG-amine-FABOC on magnetite nanoparticles.

perature, the resulting mixture was placed under a 2-Torr vacuum to remove residual trifluoroethanol from the product. The crude bis-TFEE displayed the following ^{1}H NMR spectrum: (C_6D_6) δ 4.1 (brm, 4H, CF_3CH_2-), 3.88 (s, 4H, $-COCH_2-$), 33.42 (brm, 42H, $-CH_2CH_2O-$).

To prepare the PEG silane, 20 g of PEG-ditrifluoroethylester was dissolved in 200 mL of dry toluene. The solution was heated to reflux under nitrogen utilizing a Dean−Stark apparatus to remove residual water from the solution. When the resultant solution cooled to room temperature, 6.4 mL (27 mmol) of APS was added dropwise to the PEG-ditrifluoroethylester solution under nitrogen. The resultant solution was stirred overnight under nitrogen at ambient temperature. Following the amidation reaction, the solvent was removed by distillation. After the distillation, a 0.2 Torr vacuum was applied to remove residual toluene and APS from the PEG silane. The crude half amide-ester displayed the following ^{1}H NMR spectrum: (C_6D_6) δ 7.2 (s, 1H, $-CONHCH_2-$), 4.1 (brm, 4H, CF_3CH_2-), 3.9 (s, 3H, $-COCH_2-$), 3.77 (quart., 6H, $-SiOCH_2CH_3-$, J = 7.0 Hz), 3.5 (brm, 42H, $-CH_2CH_2O-$), 3.24 (brm, 2H, $-NHCH_2CH_2-$), 1.75 (brm, 2H, $-NHCH_2CH_2CH_2-$, J = 7.6 Hz), 1.16 (trip, 9H, $-OCH_2CH_3$, J = 7.0 Hz), 0.68 (trip, 6H, $-CH_2CH_2Si-$, J = 8.6 Hz).

Synthesis of Superparamagnetic Nanoparticles. Oleyl-nanoparticles were prepared following the method by Sun[19] with modifications. First, 20 mL of phenyl ether, 0.728 g of iron (III) acetylacetonate, 2.858 g of 1,2-hexadecanediol, 2.11 mL of oleic acid (0.262 M), and 2.75 mL of oleylamine (0.198 M) were mixed in a sonicated bath. The mixture was slowly heated to 265 °C and refluxed under nitrogen for 30 min. During this process, the initial reddish-orange color of the solution gradually changed to dark black, indicative of formation of the oleyl-magnetite nanoparticles. The resultant solution was cooled to room temperature, and 250 mL ethanol was added to yield a black precipitate. The precipitate was collected from the solution with a rare earth magnet and redispersed in 50 mL of hexane to yield a clear homogeneous solution. The suspension was then precipitated with addition of 1 mL of oleic acid, 1 mL of oleylamine, and 250 mL of ethanol. To remove the oleic acid and oleylamine from the nanoparticle surface, the particles were washed in a solution of 1 M ammonium hydroxide in 1-butanol in a sonicating bath followed by washing with toluene, acetone, and ethanol.

Preparation of *t*-Boc-Protected Folic Acid. The *t*-Boc-protected folic acid was prepared by dissolving 9.25 mmol of FA in dimethyl sulfoxide (DMSO). Next, 44 mmol of di-*tert*-butyl dicarbonate (*t*-Boc) was added dropwise to the mixture and stirred for 2 h. Following the installation of the *t*-Boc protecting group, 94 mmol of *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) and 18.6 mmol of *N*-hydroxysuccinimide (NHS) were dissolved in 200 mL of DMSO, and the mixture was stirred overnight. The *t*-Boc-protected FA solution was then added into the NHS/EDAC solution to produce the succinimidal ester on the carboxyl terminus of the FA. The derivatized FA was precipitated in methylene chloride and separated by centrifugation. The product was then freeze-dried to remove the solvent.

PEG SAM Immobilization. Figure 2 shows the chemical scheme of modifying nanoparticles with TFEE silane and subsequently conjugating ethylenediamine with carboxylic acid end groups of derivatized folic acid. First, 200 mg of nanoparticles was dispersed in 100 mL of toluene in a round-bottom flask by 20 min of sonication. Following dispersion, 1 mL of the PEG-trifluoroethylester silane was added to the nanoparticle suspension, and the mixture was sonicated for 4 h at 50 °C. The resultant PEG-immobilized nanoparticle precipitate was isolated by centrifugation and washed three times with dry toluene to remove residual PEG-silane. The primary amine was created on the immobilized PEG chain termini by flooding the nanoparticle suspension with excess ethylenediamine (EDA). Next, 1 mL of EDA was added to the PEG immobilized nanoparticle suspension and allowed to react for 2 h. The particles were then isolated with a rare earth magnet and washed three times with deionized (DI) water.

Amidation of PEG Chain Termini with Folic Acid. The primary amine on the SAM terminus of the nanoparticles was amidated with the *t*-Boc-protected FA by suspending 200 mg of nanoparticles in 50 mL of DMSO along with 75 mg of *t*-Boc-protected FA. The suspension was sonicated at 60 °C for 2 h. The particles were isolated and washed

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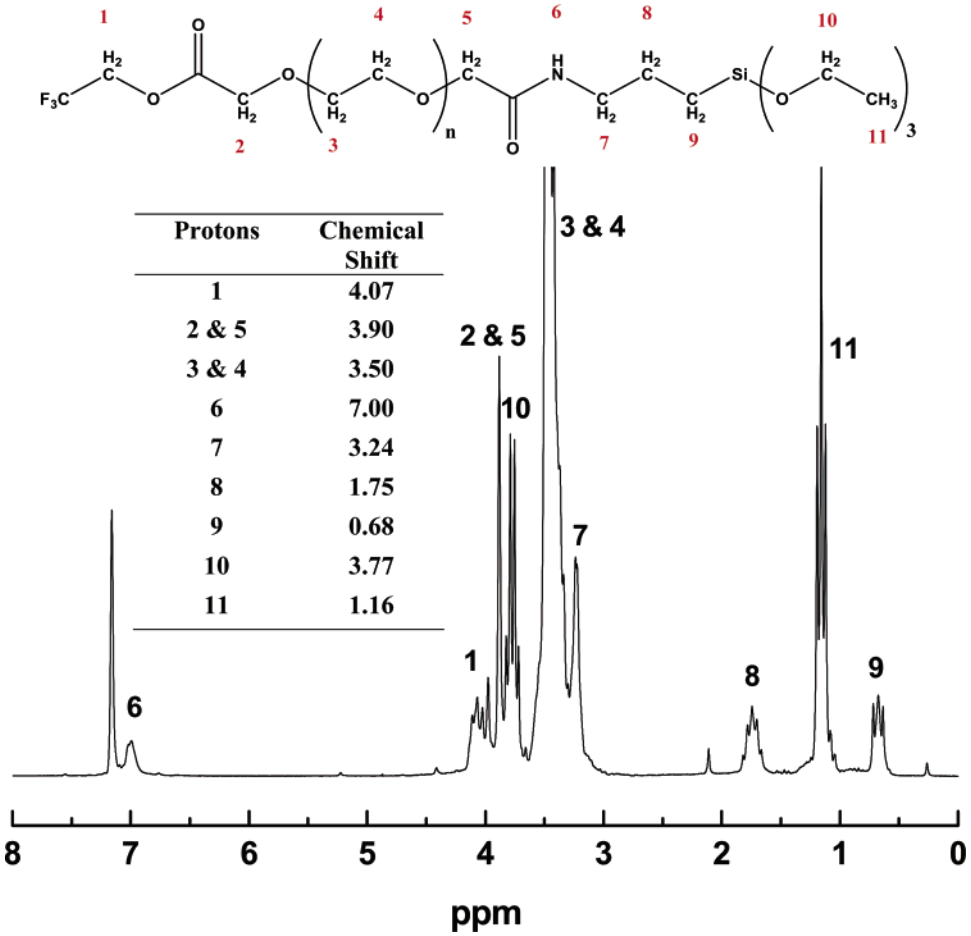

Figure 3. ^{1}H NMR spectra of the TFEE-terminal PEG silane.

twice with DMSO and three times with DI water. The particles were then resuspended in 15 mL of DI water.

To produce the carboxylic acid end groups on the PEG SAM termini, the functionalized nanoparticles were washed twice with DI water and stored overnight in DI water to hydrolyze the trifluoroethylester end group. The particles were then resuspended in 50 mL of DI water along with 88 mg of NHS and 750 mg of EDAC to produce a succinimidal ester on the termini of the PEG. The solution was sonicated for 4 h, following which the particles were isolated and resuspended in 50 mL of DMSO along with 75 mg of folic acid. The suspension was stirred for 2 h, allowing the folic acid to react fully with the PEG chain succinimidal ester terminus. The particles were then isolated, washed twice with DMSO and three times with DI water, and stored in DI water. The procedure for characterization of the *t*-Boc-protected FA has been reported elsewhere.[20]

Instrumentation. ^{1}H NMR spectra were acquired with Varian 300 MHz NMR and Bruker DPX-200 NMR spectrometers equipped with a Spectrospin 4.7 T superconducting magnet.

FTIR spectra were acquired using a Nicolet 5-DXB FTIR spectrometer with a resolution of 4 cm^{-1}. PEG-silane samples for FTIR analysis were prepared by placing a drop of the liquid between two KBr windows. To analyze the FA immobilized nanoparticles, 2 mg of dried powder of nanoparticle conjugates was mixed with 200 mg of KBr and pressed into a pellet for analysis. TEM images were acquired with a Phillips 400 TEM operating at 100 kV. Grids were prepared by dipping 300 mesh silicon-monoxide support films in the PEG-silane immobilized nanoparticle suspensions in water. The grids were then dried under vacuum for 2 h prior to analysis.

3. Results and Discussion

Surface modification of metal oxide nanoparticles is critical to ensure the biocompatibility of the nanoparticles both in vitro and in vivo. In this work, we have developed a functionalized PEG silane capable of ligand immobilization to nanoparticle surfaces. Our previous study demonstrated that the uptake of superparamagnetic nanoparticles by human breast cancer cells was increased through surface modification with PEG.[6] This increase in particle uptake was believed to be due to the high solubility of PEG in both polar and nonpolar solvents and thus the increased solubility in the cellular membrane.[21,23]

To synthesize the PEG silane, a PEG diacid was first converted to an acid chloride that was converted into a bistrifluoroethylester (TFEE), as shown in Figure 1. By using 3-aminopropyltriethoxy silane (APS), we formed a PEG silane with increased molecular stability through hydrogen bonding between carbonyl and amine groups across the particle surface. The structure of the TFEE terminal PEG silane was identified by ^{1}H NMR analysis. The chemical shifts for protons from position 1 to 11 in the TEFE terminal PEG silane are shown in Figure 3 and its inset table. The molecular structure of the TFEE-terminal PEG silane was confirmed by the presence of all characteristic peaks and peak integration.

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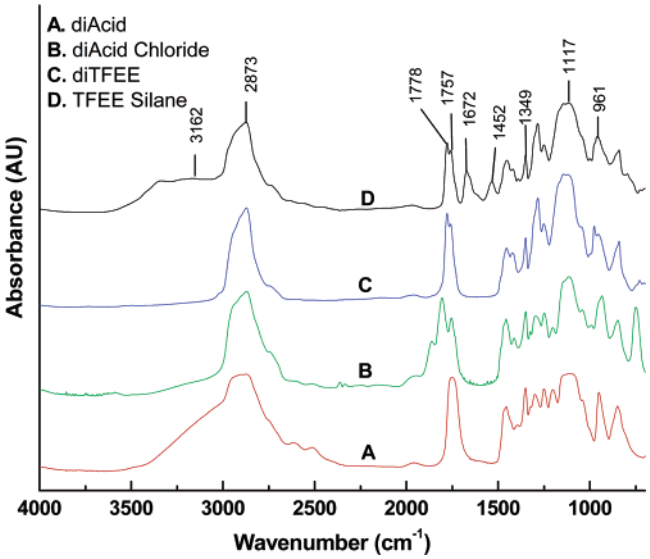

Figure 4. FTIR spectra of (A) PEG-bisdiacid, (B) PEG-acid chloride, (C) PEG-trifluoroethylester, and (D) PEG-trifluoroethylester terminal-silane.

To further verify the successful synthesis of the TFEE terminal PEG silane, FTIR spectra were also collected at each step of the synthesis, as shown in Figure 4. The broad band at 3200 cm^{-1} corresponds to −OH groups in the dicarboxylic acid structure (A) and was lost following the conversion of PEG diacid to the acid chloride (B), and, as expected, the carbonyl stretch is shifted to higher frequency (above 1800 cm^{-1}) and showed greater complexity as a result of substitution of the −OH group with the chlorine group. Conversion of diacid chloride to bis-TFEE (C) collapses the carbonyl band to a simpler structure at about 1760 cm^{-1}, typical of an electron-deficient ester carbonyl. The wide −OH stretch band above 3000 cm^{-1} in the PEG diacid is absent in both the acid chloride and the TFEE spectra. Evidence for the half-amide/ester structure (D) is found in the final FTIR spectrum in which both the TFEE ester and the amide carbonyl peaks are clearly seen (1778 and 1672 cm^{-1}, respectively). Identification of the TFEE end groups on the molecule was unreliable due to the peak overlap between the PEG ether groups and the C−F stretch groups. The broad band at 3162 cm^{-1} resulted from the −NH stretch band in the amide linkage between the silane and the PEG (D).

Once the synthesis of the TFEE terminal PEG silane had been confirmed with ^1H NMR and FTIR spectroscopy, we immobilized the PEG terminal TFEE silane on the nanoparticles following the scheme outlined in Figure 2. To demonstrate the functionality of the newly synthesized TFEE terminal PEG silane, we chose to convert the TFEE end group to a primary amine, allowing *t*-Boc-protected FA to be immobilized on the primary amine via the two carboxylic acid groups present on the glutamic acid residue. FTIR spectra confirmed the successful surface modification as shown in Figure 5. The $-CH_2$ stretching vibrations present on native nanoparticles (A) are seen to increase in intensity following the attachment of the PEG-based SAM onto the nanoparticle (B). Following the FA-*t*-Boc immobilization, the amide carbonyl bands at 1642 and 1546 cm^{-1} were intensified (C).

To further demonstrate the conjugating flexibility of the TFEE terminal PEG silane, FA was grafted onto the PEG chain terminus through amidation between the TFEE end group and

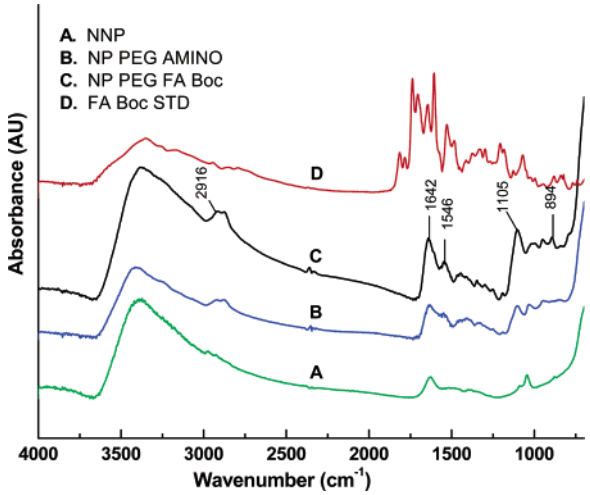

Figure 5. Surface modification of nanoparticles with folic acid (FA) through the carboxylic acid groups. FTIR spectra of (A) native (uncoated) nanoparticles, (B) nanoparticles immobilized with PEG-carboxyl terminal SAM, (C) *t*-Boc-FA grafted nanoparticles, and (D) pure *t*-Boc-FA-NHS.

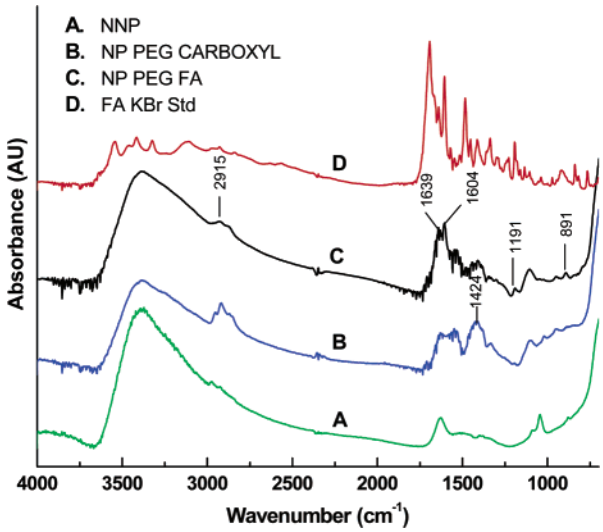

Figure 6. Surface modification of nanoparticles with FA via amine groups on the pteridine ring. FTIR spectra of (A) native (uncoated) nanoparticles, (B) nanoparticles immobilized with PEG-carboxyl terminal SAM, (C) FA grafted nanoparticles, and (D) pure FA.

primary amine groups present on the pteridine rings of FA. The TFEE chain termini are capable of reacting with primary amines in organic solvents and aqueous solvents through either the conversion of the TFEE to a primary amine or the hydrolysis of the TFEE terminal group to the corresponding carboxylic acid in water. The free carboxylic acid is then esterified to a succinimidal ester allowing aqueous amidation of FA. FTIR analysis of the aqueous amidation of FA is shown in Figure 6. Following the surface modification with the PEG silane, the $-CH_2$ stretching vibrations at 2900 cm^{-1} increased in absorbance, indicating the presence of the PEG silane on the particle surface (B). The broad peak between 1500 and 1600 cm^{-1} in the spectrum is consistent with the carboxylate intermediate. Following FA immobilization, the primary amide peaks increased in absorbance at 1639 and 1604 cm^{-1} (C), consistent with the bonding of the FA molecule to the PEG SAM terminus.

TEM images were taken to characterize the dispersivity of nanoparticles before and after surface modification. The addition

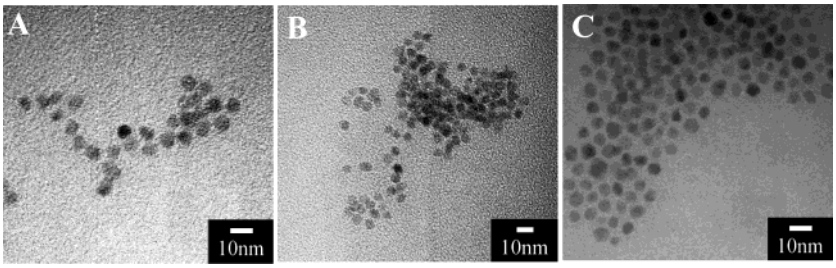

Figure 7. TEM micrographs of nanoparticles modified with (A) TFEE terminal PEG silane, (B) amine-terminated PEG, and (C) FA-*t*-BOC-terminal PEG silane.

of APS to the bistrifluoroethylester PEG shown at step D in Figure 1 was statistical, which might lead to the formation of three types of PEG products: (1) PEG molecules in solution that have not reacted with APS, (2) PEG molecules in solution whose ends have reacted with APS (bis-APS), and (3) bifunctional PEG molecules with both APS and TFEE end groups. Residual PEG bistrifluoroethylester molecules in solution will not react with the nanoparticle during the surface modification due to the lack of amine groups on the nanoparticle surface. The TEM image in Figure 7A shows that nanoparticles modified with TFEE terminal PEG are well dispersed. This result suggests that the bifunctional PEG molecules were the dominant product during the process of the addition of APS to the bistrifluoroethylester PEG. This is because if the bis-APS PEG silanes were a dominant product, nanoparticles modified with bis-APS silanes may cross-link other nanoparticles and form nanoparticle agglomerates. The TEM image in Figure 7B shows the dispersion of ethylenediamine-modified nanoparticles. Dispersion of amine terminal PEG silane nanoparticles in toluene is reduced due to the reduction of solubility of the nanoparticles with primary amine groups in the nonpolar solvent. However, following amidation, nanoparticle dispersion is maintained. Dispersion following the immobilization of the FA-*t*-BOC on the amine terminal PEG silane is also confirmed, as shown in Figure 7C.

Butterworth and colleagues have shown that nanoparticle dispersion is a function of PEG molecular weight. Either higher (e.g., $> \sim 5000$) or lower (e.g., $< \sim 350$) molecular weight would give rise to high particle stability and dispersivity. High molecular weight can provide complete PEG coverage on nanoparticle surfaces through chains laying across the entire nanoparticle surface. Alternately, low molecular weight can improve particle dispersion as a result of higher monolayer packing densities that would increase steric hindrance.[22] In this study, we used a 600 molecular weight PEG polymer capable of high-density grafting. Immobilizing a lower molecular weight PEG SAM on the nanoparticle surface also results in more available functional chain termini, that is, more amidation sites to which targeting ligands may be grafted. In addition, APS at the base of the molecule can enhance the stability of SAM on the nanoparticle surface.

The TFEE end group provides a reactive carboxylic acid group capable of amidation through the immobilization of EDA or folic acid on nanoparticles. This reactive, dual-functional end group allows successful immobilization of molecules containing either primary amine or carboxylic acid groups, thus improving the compatibility of the PEG SAM with prospective targeting agents.

4. Conclusions

We have devised a new functional PEG silane that can be self-assembled on metal oxide colloidal systems to conjugate with cell targeting agents that have either carboxylic or amine terminal groups. The system takes advantage of common carboxylate PEGs and 3-aminopropyltriethyoxysilane, which increases the stability of the PEG monolayer on the nanoparticle surface as a result of formation of hydrogen bonding between PEG interchains. Amidation of PEG termini has been demonstrated by immobilizing FA on the termini of the PEG molecule. By immobilizing FA onto the PEG chain termini via either carboxylic acid or amine groups, the flexibility of this PEG silane system has been established for subsequent immobilization of intended targeting agents. Further, trifluoroethylester-PEG silane amidation is compatible with both organic and aqueous solvents. The trifluoroethylester end group provides a stable leaving group for amidation in organic solvents. Aqueous hydrolization to the corresponding carboxylic acid can be converted to a succimidal ester, a stable leaving group for peptide immobilization in aqueous systems.[23] The compatibility of TFEE-PEG silane amidation with aqueous solvent allows simple immobilization of ligand systems compatible with aqueous solvents alone such as proteins. In addition, PEG silanes with different molecules can be made by converting available diol PEGs with different molecular weights to dicarboxylic acids.

Metal oxide nanoparticles are becoming increasingly important in medicine for MRI contrast enhancement and controlled drug delivery. This functional PEG silane allows covalent immobilization of PEG to the nanoparticle surface to increase particle circulation time in blood and particulate dispersion. In addition, this new monolayer is capable of ligand grafting through amidation, allowing targeted uptake of the nanoparticle ligand system into tumors in vivo. The functionality of this monolayer allows virtually any targeting agent to be immobilized to a nanoparticle surface provided it has either a free carboxylic acid or a primary amine capable of amidation.

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