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Cancer-Specific Gene Silencing through Therapeutic siRNA Delivery with B Vitamin-Based Nanoassembled Low-Molecular-Weight Hydrogelators

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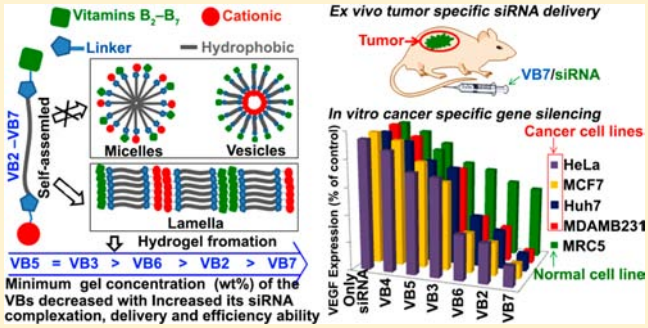
Supporting Information

ABSTRACT: This paper describes the synthesis, characterization, and *in vitro* and *in vivo* siRNA transfection ability of B vitamin-based cationic clickable bolaamphiphiles (VBs). Our VBs derived from vitamins B_2, B_3, B_5, B_6, and B_7 formed nanoassembled low-molecular-weight hydrogelators (LMWGs, vitagels). The vitagels **VB2**, **VB6**, and **VB7** (derived from vitamins B_2, B_6, and B_7, respectively) facilitated delivery of small interfering RNAs (siRNA), efficiently silencing gene expression specifically into cancer cell lines; in addition, the LMWGs derived from vitamins B_3, B_5, and B_6 were biocompatible. An *ex vivo* study in a mouse model revealed that the siRNA delivered by the vitagel **VB7** was located primarily at the site of the tumor. The gene silencing efficiency of vascular endothelial growth factor siRNA delivered by vitagels was dependent on the nature of the vitamin headgroup, the N/P ratio, and, interestingly, the hydrogelation properties of the VBs.



INTRODUCTION

The specificity and potency of synthetic small interfering RNAs (siRNAs) as a means of regulating gene expression is a promising approach for the treatment of incurable diseases and genetic disorders, including cancers.[1] An efficient delivery system is a necessity for any successful siRNA therapy.[2−4] The development of target-specific siRNA (drug) delivery systems [so-called "smart" drug delivery systems (DDSs)] remains a challenge both academically and for the modern pharmaceutical industry. An effective DDS should decrease (i) the frequency of the dosage taken a patient, (ii) fluctuations in the levels of the circulating drug, and (iii) drug side effects. Various forms of nanocarriers are being used widely in targeted DDSs,[5−10] including lipid-like materials,[11] inorganic nanoparticles,[12,13] peptides,[13,14] and polymeric materials.[15−18]

Vitamins are essential biomolecules; they are, however, overexpressed on the surfaces of cancer cells, relative to normal cells.[18−21] Thus, their specific receptor or carrier-mediated entry can be utilized as a biological mechanism for the delivery of therapeutic siRNAs into cells. Because hydrogels are typically biocompatible, they are suitable for use in a wide range of applications.[22−33] For example, low-molecular-weight hydrogelators (LMWGs) can be employed as alternatives[22−25] for polymeric gels (P-gels) in many applications.[26−33] Recently, P-gels have been reported for the controlled release of siRNA.[30−32]

In this study, we developed LMWG-based cancer-targeting siRNA delivery systems (vitagels) containing, as targeting ligands, cationic lipids coupled with B vitamins. We demonstrate herein that therapeutic siRNA delivery with the vitagels **VB2**, **VB6**, and **VB7** can efficiently silence gene expression, specifically into cancer cell lines.

RESULTS AND DISCUSSION

Synthesis of B Vitamin-Based Cationic Clickable Bolaamphiphiles (VBs). We prepared VBs featuring amino (cationic, polar) head groups and hydrophobic chains associated with vitamins B_2−B_7 (neutral, polar) through amide, carbamate, and triazole linkers (Figure 1). Coupling of the acetylenated vitamin B derivatives **2c**,**d**,**f**−**h** with the azide derivative of a cationic lipid (**1b**), through click chemistry, provided the precursors **VB2″**, **VB3′**, **VB5′**, **VB6′**, and **VB7′**, respectively; in addition, coupling of adenine ("vitamin B_4", **2e**) with the carboxylic acid derivative of the cationic lipid (**1d**), through peptide chemistry, provided the precursor **VB4′**; subsequent deprotection of the various protecting groups provided the desired compounds **VB2**−**VB7** (Supporting Information Figure S1 and Scheme 1).

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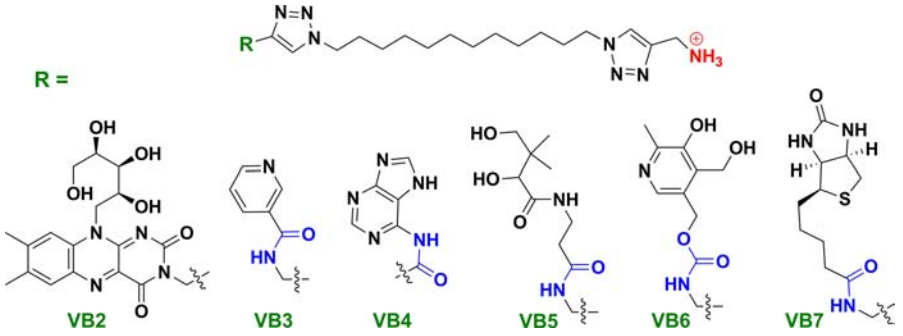
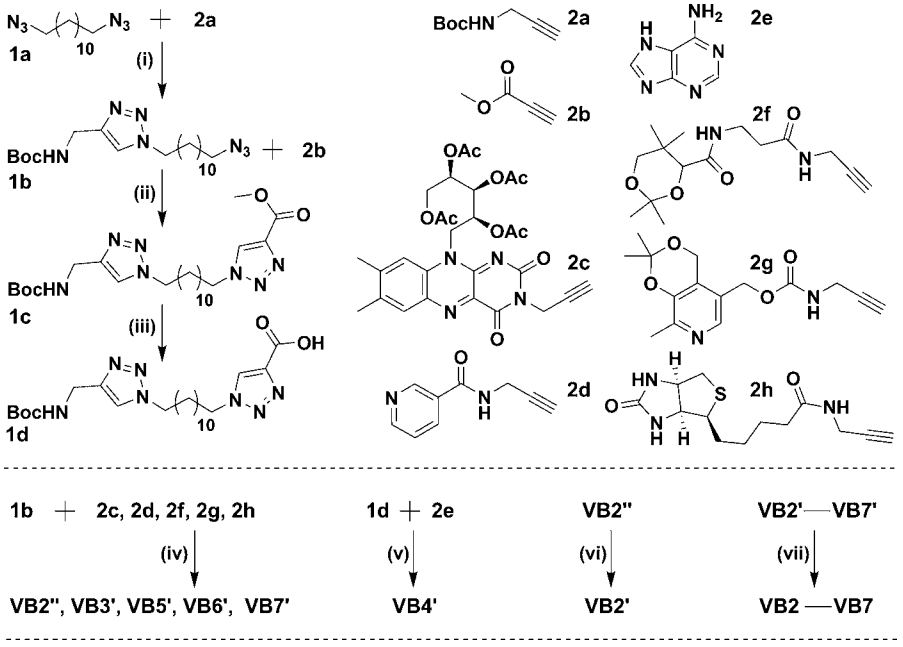


Figure 1. Chemical structures of the VBs.

Scheme 1. Synthesis of VBs[a]



[a](i), (ii), and (iv) $CuSO_4$, sodium ascorbate, THF/*t*-BuOH/H_2O (2:2:1), 45 °C, 24−48 h; (iii) aq. 2 N NaOH, methanol, rt, 6 h; (v) TBTU, DIPEA, DMF, rt, 24 h; (vi) 7 N NH_3, methanol, rt, 48 h; (vii) 4 M HCl in dioxane, rt, 6−8 h.

Cytotoxicity Assay. We used the WST-1 assay to assess the toxicity of the VBs with siRNA at different N/P ratios (7.5:1 and 12.5:1) or molar ratios (300:1 and 500:1). Figure 2 reveals that these VBs did not exhibit significant toxicity at their optimal concentrations for transfection in any type of cell line. Approximately 85% of the cells survived at each concentration of the VBs. Notably, the cytotoxicity of each VB was lower than that of the commercially available siRNA transfection reagent Lipofectamine2000. Accordingly, these VBs appeared to be highly biocompatible materials.

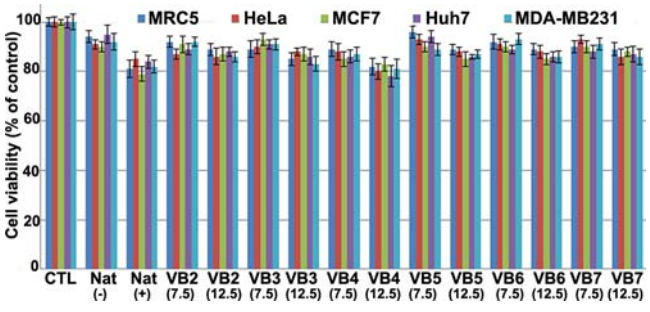


Figure 2. Cytotoxicity assays of Fl−VEGF−siRNA (100 nM) with **VB2**−**VB7** at molar ratios of 300:1 and 500:1 [CTL: untreated cell; Nat(−): only siRNA; Nat(+): siRNA with lipofectamine2000; $N = 3$].

siRNA-Binding Ability: Gel Retardation Assays. The vitagels **VB2**, **VB3**, **VB5**, **VB6**, and **VB7** underwent complexation with the siRNA at N/P ratios of 5, 7, 7, 6, and 4, respectively; in contrast, **VB4** did not undergo complexation with the siRNA even at an N/P ratio of 8 (Figure 3). These findings suggested that supramolecular hydrogelation of the VBs resulted in their displaying polymer-like behavior, which helped in the complexation of the siRNA.

Hydrogel Formation: Transmission Electron Microscopy. The vitagels **VB2** and **VB7** formed transparent hydrogels in pure water at concentrations of 1.8 and 0.5 wt %, respectively, while **VB3**, **VB5**, and **VB6** formed opaque hydrogels at concentrations of 3, 3, and 2 wt %, respectively (Figure 4A); in contrast, **VB4** was insoluble in aqueous solutions, even at lower concentrations. These vitagels were all soluble in acidic aqueous solutions, but in basic media they precipitated (data not shown). To the best of our knowledge, the LMWGs we prepared from the vitamin B_3, B_5, and B_6 derivatives are the first such species to be reported; vitamin B_6-

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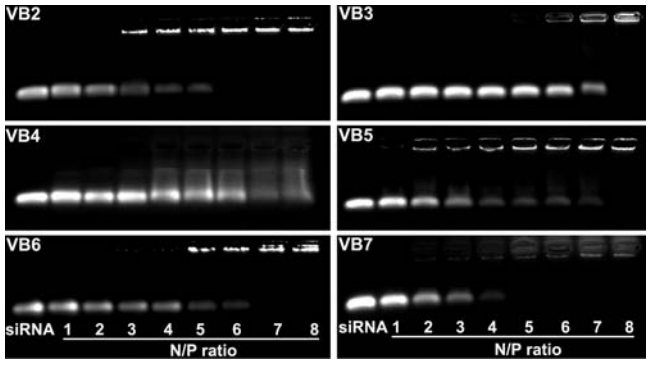

Figure 3. Gel retardation assays of **VB2**–**VB7** with the siRNA at various N/P ratios.

based polymeric hydrogels have, however, been reported previously.[21] The biocompatibility of our LMWG biomaterials suggests that they might be suitable for use in a wide range of applications.

We used transmission electron microscopy (TEM) to investigate the supramolecular structures formed by the xerogels of the vitagels in the presence and absence of siRNA. The TEM images of the xerogels of the vitagels alone revealed long, extended, entangled helical nanofibers, similar to the extracellular matrices found in tissue (Figure 4B,D–G and Supporting Information Figures S2–S4).[33] In contrast, the xerogels of the vitagel/siRNA mixtures (500:1 molar ratio) comprised small particles of siRNA condensed with nanofibers (Figure 4C,H–K). The TEM data of the vitagels also suggested that they formed lamellar-type structures (abstract figure),[23,26] which can help in the efficient complexation and delivery of siRNA.[34] Thus, our vitagels might also have further potential utility in tissue engineering, treating infectious wounds, and cosmetics, as well as antiseptic matrices.

***In Vitro* siRNA Internalization.** We used a cellular uptake assay to examine the internalization of a fluorescein-tagged vascular endothelial growth factor (VEGF) siRNA (Fl–VEGF–siRNA; Figure 5). The vitagels **VB2**, **VB6**, and **VB7** were approximately two to three times more efficient at delivering the siRNA (100 nM) at N/P ratios of 7.5:1 and 12.5:1 into cancer cell lines (HeLa, MCF7, Huh7, MDA-MB-231) than they were into a normal cell line (MRC5). In contrast, **VB3**, **VB5**, and **VB4** did not display any such selectivity for internalization of the siRNA under otherwise identical conditions. In addition, the vitagel **VB7**/siRNA (500:1) provided cellular uptake, specifically into the cancer cell lines,

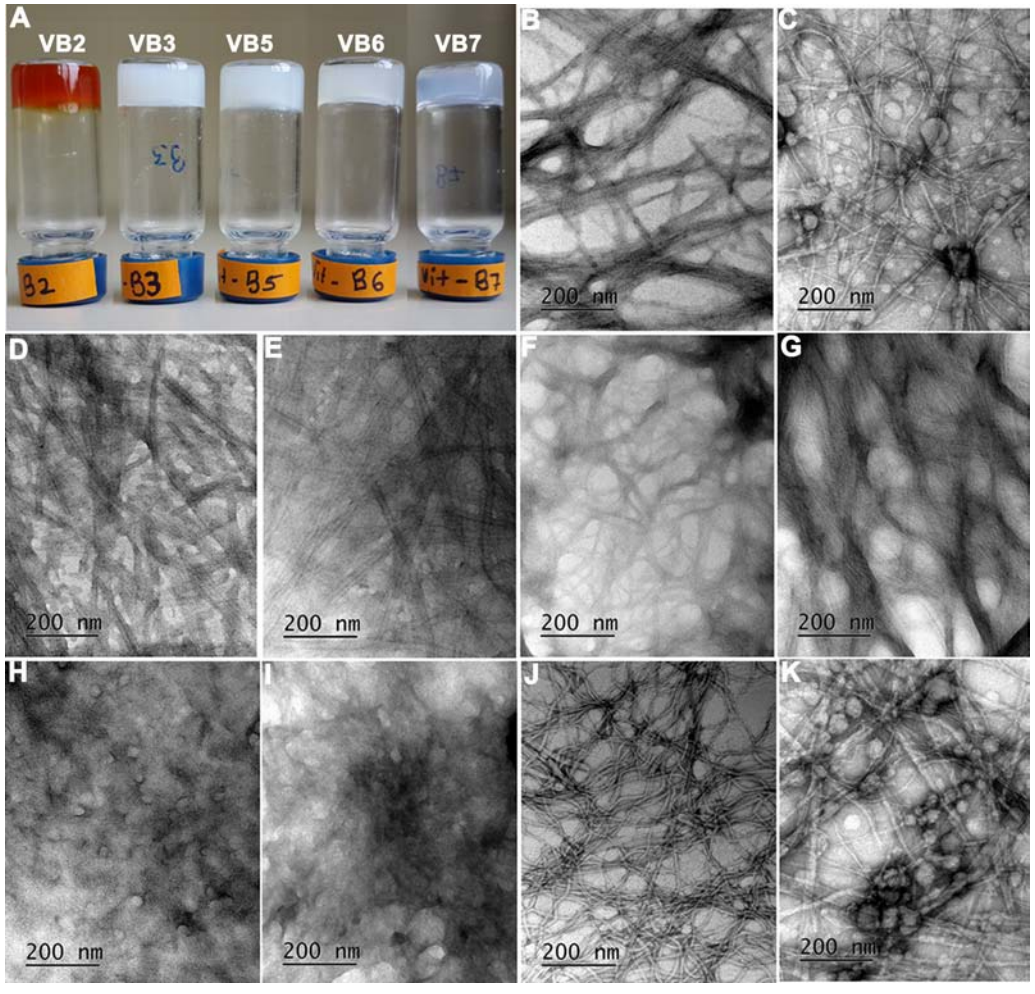

Figure 4. (A) Photograph of the vitagels **VB2**, **VB3**, **VB5**, **VB6**, and **VB7** in water at concentrations of 1.8, 3.0, 3.0, 2.0, and 0.5 wt %. (B–K) TEM images of (B) **VB2**, (C) **VB2**/siRNA, (D) **VB3**, (E) **VB5**, (F) **VB6**, (G) **VB7**, (H) **VB3**/siRNA, (I) **VB5**/siRNA, (J) **VB6**/siRNA, and (K) **VB7**/siRNA.

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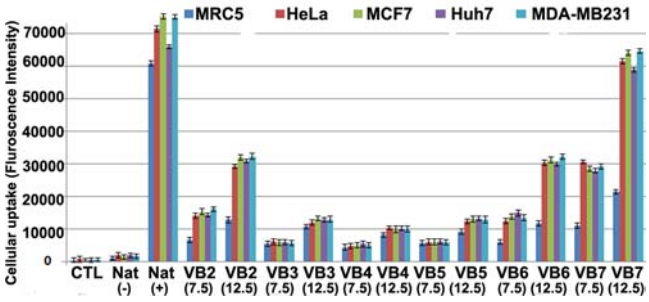

Figure 5. Cellular uptake of Fl−VEGF−siRNA (100 nM) with **VB2**−**VB7** at N/P ratios of 7.5:1 and 12.5:1; CTL: untreated cells; Nat(−): only siRNA; Nat(+): siRNA with Lipofectamine2000; $N = 3$.

that was almost equal to that of Lipofectamine2000 at an siRNA concentration of 100 nM.

Confocal Microscopy. We employed confocal microscopy to study the localization of Fl−VEGF−siRNA in the MDA-MB-231 cells. We formulated each VB with Fl−VEGF−siRNA (100 nM) at a molar ratio of 500:1. Figure 6 reveals the absence of green fluorescence in the cells treated with naked siRNA, confirming the lack of siRNA uptake. Similar to our results from the cellular uptake assays, confocal microscopy revealed that the cellular uptake mediated by the vitagels **VB2**, **VB6**, and **VB7** was superior to those of **VB4** and the naked siRNA. Although **VB4** had a chemical structure similar to those of the other vitagels, it did not mediate any prominent siRNA internalization. These observations suggest that the siRNA delivery ability of these VBs was dependent on their hydrogelation properties. When Fl−VEGF−siRNA was delivered by Lipofectamine2000, it appeared that siRNA accumulated in the endosome (punctuate pattern), with localization in the cytosol as well as the nuclei of the cell, similar to that provided by most particulate carriers.[2−4] When we used our vitagels to deliver Fl−VEGF−siRNA, the siRNA did not accumulate in the endosome (uniform pattern or diffuse green fluorescence in cytoplasm), but rather underwent significant localization in the cytosol of the cell, similar to the situation when using polymer-based nanomaterials as transfection agents.[31]

Drug Inhibition Assays and ELISA Assays. To study the internalization mechanism of Fl−VEGF−siRNA mediated by the vitagel **VB7**, we preincubated MCF7 cells with metabolic and endocytic inhibitors and analyzed the systems through cellular uptake assays (Figure 7A).[35] Approximately 76% of the internalization of siRNA/**VB7** was inhibited after incubation with sodium azide/2-deoxyglucose, indicating that siRNA/**VB7** internalization is an energy-dependent process. Approximately 45% of the uptake of siRNA/**VB7** was inhibited after incubation with methyl-β-cyclodextrin, which affects both clathrin- and caveolae-mediated pathways. We found that preincubation with chlorpromazine significantly inhibited (by 68%) the internalization of siRNA/**VB7**, suggesting that clathrin-mediated endocytosis may be a one of internalization pathways for siRNA/**VB7**. The presence of genistein did not affect the internalization of siRNA/**VB7**, suggesting that the uptake of siRNA/**VB7** does not occur through caveolae-mediated endocytosis. Preincubation with amilorides significantly inhibited (by 64%) the internalization of siRNA/**VB7**; thus, we suspected that macropinocytosis might be a internalization pathway for siRNA/**VB7**. Further study will be necessary to confirm the internalization mechanism of siRNA mediated by such vitagels.

Next, we performed an ELISA assay to investigate the degree of VEGF protein expression (Figure 7B). We assessed the siRNA transfection results after 24 h, using an N/P ratio of **VB2**−**VB7** to siRNA of 12.5:1. The highest degrees of gene knockdown, approximately 85%, 75%, and 65%, occurred specifically into the cancer (HeLa, MCF7, Huh7, MDA-MB-231) cell lines in the presence of the vitagels **VB7**, **VB2**, and **VB6** and siRNA (100 nM; 500:1 molar ratio); in contrast, approximately 35%, 28%, and 30% gene knockdown occurred into the normal cell line (MRC5) under otherwise identical conditions. In addition, no gene knockdown occurred from the scrambled sequence of VEGF−siRNA under otherwise identical conditions. To the best of our knowledge, this report is the first to describe (i) siRNA delivery by LMWGs based on derivatives of vitamins B_6 and B_7 and (ii) two to three times more efficient delivery of the siRNA and gene knockdown occurring specifically into cancer cell lines, relative to normal cells, when applying LMWGs based on vitamins B_2, B_6, and B_7 at an N/P ratio of 12.5:1 with an siRNA concentration of 100 nM. Notably, however, vitamin B_2-, B_6-, and B_7-based nanoparticles and polymers have been reported for the delivery of drugs, including genes, into cancer cells.[19−21]

siRNA-Complexation Stability in Fetal Bovine Serum (FBS). We used gel electrophoresis to examine the stability of our vitagel/siRNA complexes in 60% FBS over different time intervals at 37 °C (Figure 8). The degradation of the dissociated siRNA was negligible in FBS. Upon treatment with heparin, the intact siRNA dissociated from the vitagel/siRNA complexes; in contrast, we detected no intact siRNA from the naked siRNA. Taken together, these findings indicate that our vitagels could effectively prevent the siRNA from undergoing enzymatic degradation in the serum.

RT-PCR and *Ex Vivo* Internalization. We used RT-PCR experiments, executed using agarose gel electrophoresis and staining with ethidium bromide, to confirm the efficacy of siRNA delivery by our vitagels **VB7**, **VB2**, and **VB6** (Figure 9A). For these studies, we mixed siRNA (100 nM) with the vitagels **VB2**, **VB6**, and **VB7**, each at an N/P ratio of 12.5:1. The presence of siRNA alone did not affect the quantity of mRNA. In contrast, treatment of the cancer cell lines with siRNA in the presence of **VB2**, **VB6**, or **VB7** significantly decreased the production of mRNA.

Among our tested vitagels, **VB7** displayed the best siRNA transfection ability *in vitro*. To assess its potential utility as an siRNA delivery agent, we performed *in vivo* experiments of **VB7** with tumor-bearing athymic nude mice (Figure 9B). We injected MDA-MB-231 cells (5 × 106 cells/mouse) subcutaneously into the right flank of a female mouse and then delivered Fl−VEGF−siRNA (0.1 mg/kg) and siRNA/**VB7** (molar ratio: 1:500) systemically through tail-vein injections. The mouse was then sacrificed through cervical dislocation, and its organs extracted. Fluorescence images were detected within a minute with *ex vivo* imaging system (IVIS spectrum, PerkinElmer, USA). The fluorescence signals of the major organs (liver, spleen, kidney) were normalized to their weights. Within a time course of 3 h, we compared the siRNA uptake mediated by the vitagel **VB7** within the tumor with those of the other major organs. The results in Figure 9B are similar to those from our *in vitro* study, suggesting that the vitagel **VB7** has potential utility as an siRNA delivery agent specifically into

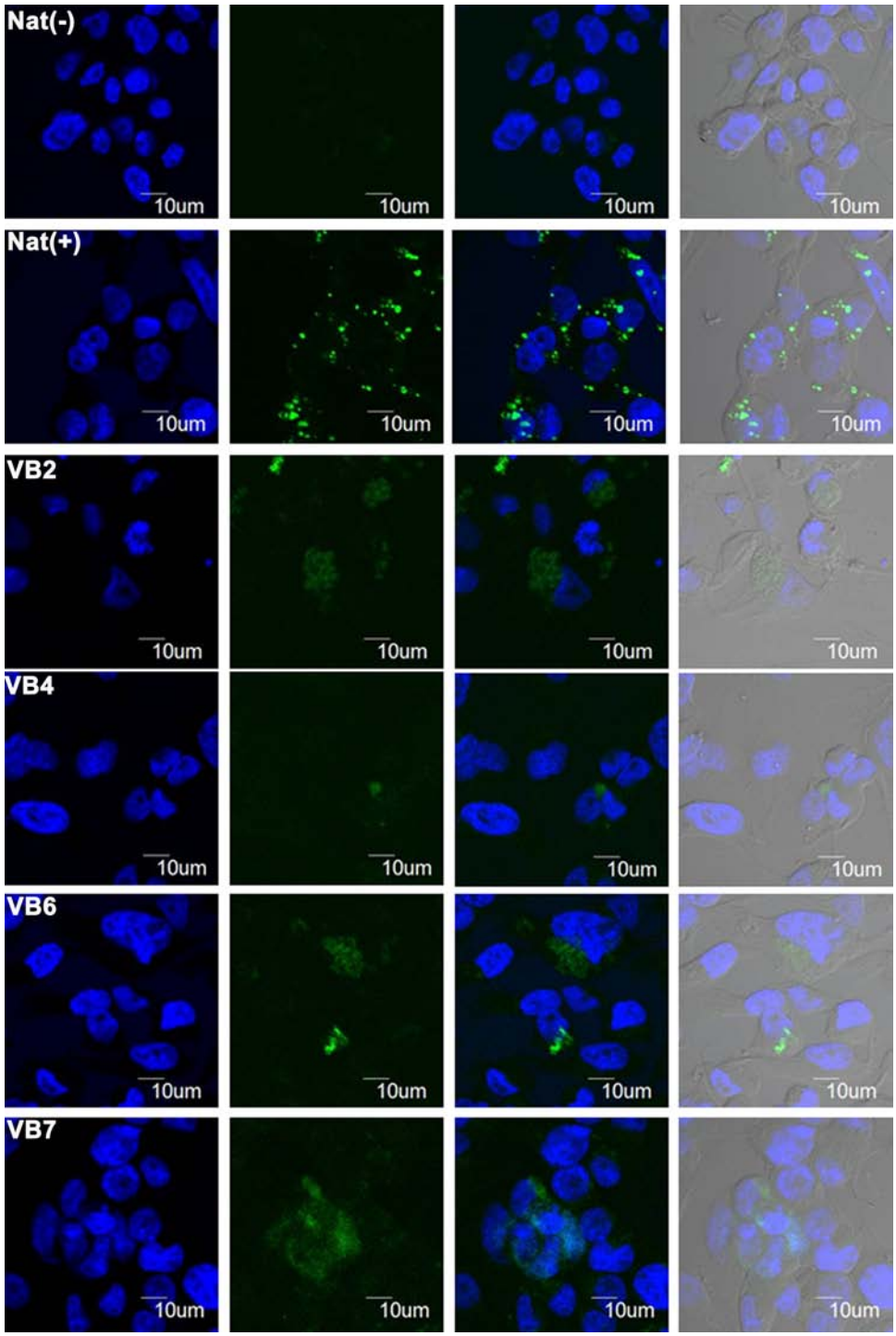

Figure 6. Confocal microscopy images of live cells that had been treated with Fl−VEGF−siRNA (100 nM) and **VB2**, **VB4**, **VB6**, and **VB7** at molar ratios of 500:1; CTL: untreated cells; Nat(−): only siRNA; Nat(+): siRNA with Lipofectamine2000; N = 3. Scale bars: 10 μm.

cancer cells. Additional *in vivo* studies of the efficacy of using vitagels for siRNA delivery are currently in progress.

■ CONCLUSION

We have synthesized a series of VBs (**VB2**−**VB7**) from vitamins B_2−B_7, respectively. In the case of **VB3**−**VB7**, the vitamins were covalently linked through biodegradable (peptide, carbamate) linkers, potentially allowing them to act as provitamins. All of our VBs formed supramolecular hydrogelators, except for **VB4**. We found that clathrin-mediated endocytosis and macropinocytosis were possible internalization pathways for the **VB7**/siRNA complex. An *ex vivo* study revealed that the siRNA delivered by the vitagel **VB7** was located specifically at the tumor of the mouse, relative to the other major organs. Our study is, to the best of our knowledge, the first of its kind in which a therapeutic siRNA, delivered with LMWGs derived from vitamins B_2, B_6, and B_7, can be used to efficiently silence gene expression specifically in cancer cells, relative to normal cells; in addition, it is the first to report biocompatible LMWGs derived from vitamins B_3, B_5, and B_6.

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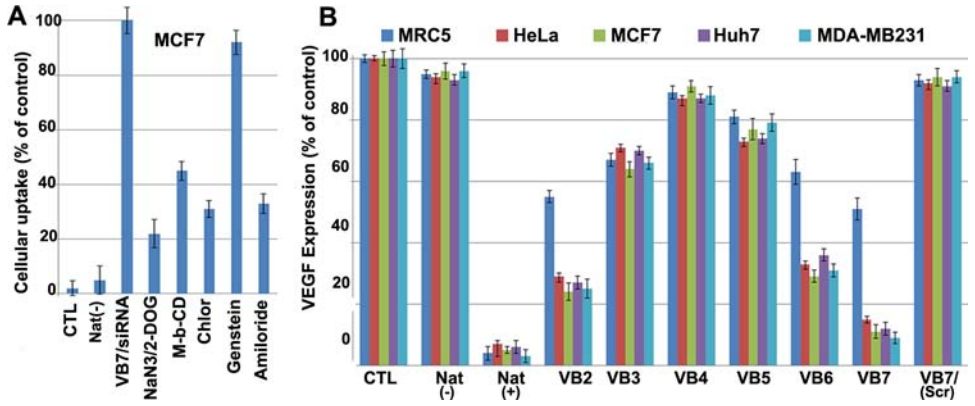

Figure 7. (A) Drug inhibition assay of Fl−VEGF−siRNA (100 nM) with **VB7** at a molar ratio of 500:1. (B) ELISAs of VEGF−siRNA (100 nM) with **VB2**−**VB7** at a molar ratio of 500:1; CTL: untreated cells; Nat(−): only siRNA; NaN_3/2-DOG: sodium azide with 2-deoxyglucose; M-β-CD: methyl-β-cyclodextrin; Chlor: chlorpromazine; Nat(+): VEGF−siRNA with lipofectamine2000; Scr: scrambled VEGF−siRNA; N = 3.

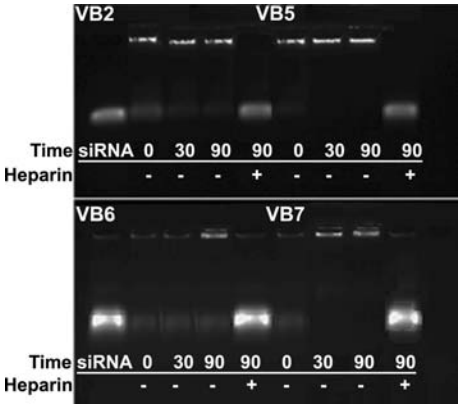

Figure 8. Stability of the complexes formed between **VB2**, **VB5**, **VB6**, **VB7** and the siRNA at N/P ratios 6 over different time intervals in the presence of FBS.

We suggest improving the hydrogelation ability (i.e., lower the minimum gel concentration) of the LMWG through optimization of chemical structures to further enhance the efficiency of LMWG-based siRNA delivery systems. Finally, targeted DDSs are among the most promising means of delivering chemotherapeutic drugs; vitamin-based LMWGs have great potential for use in tumor-targeting DDSs, as well as in various biomedical applications.

■ EXPERIMENTAL PROCEDURES

General Experimental Details. All chemicals were purchased from Sigma−Aldrich, Fluka, TCI, Lancaster, Proligo, or Glen Research, and used without further purification. All reactions were performed in flame-dried glassware under argon Flash column chromatography was performed using Merck silica gel 60 (230−400 mesh). Melting points were determined using an Electrothermal IA9100 apparatus. High-resolution (HR) fast atom bombardment (FAB) mass spectra were

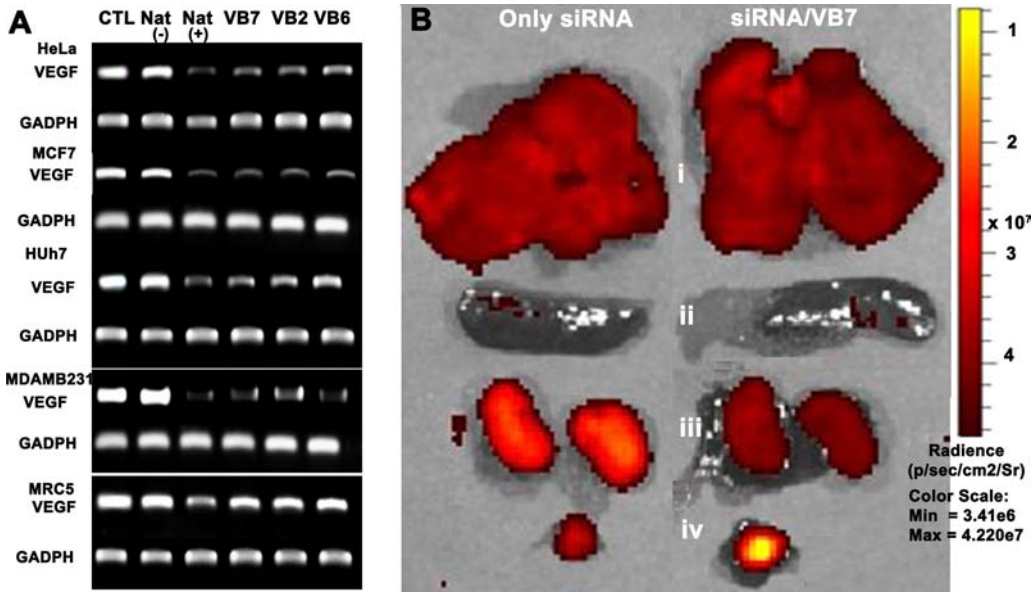

Figure 9. (A) RT-PCR experiment of VEGF−siRNA (100 nM) with **VB2**, **VB6**, and **VB7** at a molar ratio of 500:1; CTL: untreated cell; Nat(−): only siRNA; Nat(+): siRNA with Lipofectamine2000 (N = 3). (B) *Ex vivo* IVIS images of the (i) liver, (ii) spleen, (iii) kidney, and (iv) tumor (MDA-MB-231) from a nude mouse treated with (left) Fl−VEGF−siRNA alone and (right) Fl−VEGF−siRNA with **VB7** at a molar ratio of 500:1. The right-hand bar displays the intensity of the fluorescence signal from Fl−VEGF−siRNA.

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recorded using a Jeol JMS700 HR mass spectrometer at the Korea Basic Science Center, Daegu, Korea. ^{1}H and ^{13}C NMR spectra were recorded using an FT-300 and FT-600 MHz Bruker Aspect spectrometer. Chemical shifts are reported in parts per million (ppm) downfield relative to the internal standard, tetramethylsilane (TMS). Coupling constants are reported in hertz (Hz). Spectral splitting patterns are designed as follows: s, singlet; d, doublet; dd, double doublet; ds, distorted doublet; bs, broad singlet; dt, distorted triplet; t, triplet; m, multiplet; br, broad.

siRNA Sequences. The following siRNA sequences were purchased from M-biotech, South Korea:

Antisense strand (AS) VEGF–siRNA: 5′- GAU CUC AUC AGG GUA CUC CdTdT

Sense strand (SS) VEGF–siRNA: 5′- GGA GUA CCC UGA UGA GAU CdTdT

Fluorescein-tagged AS VEGF–siRNA: 5′- 5,6-FAMGAU CUC AUC AGG GUA CUC CdTdT

Scrambled AS VEGF–siRNA: 5′- AAA UUC CCG CGU UAC GCG UdTdT

Scrambled SS VEGF–siRNA: 5′ - ACG CGU AAC GCG GGA AUU UdTdT

Hydrogel Formation. Hydrogels were prepared by dissolving **VB2**, **VB3**, **VB5**, **VB6**, and **VB7** at 1.8, 3.0, 3.0, 2.0, and 0.5 wt %, respectively, in distilled water. After gentle heating (up to 80 °C) and cooling at room temperature, the complete volume of water became immobilized and could keep its own weight. Hydrogel formation was confirmed by turning each vial upside down. All of the hydrogels of VBs were stable for at least 4 weeks at room temperature.

TEM. Solution of a vitagel (3 mg/mL) or a mixture of siRNA and a vitagel (molar ratio: 1:500) was placed on copper Formvar/carbon-coated grid, air-dried for 2 min, negatively stained with uranyl acetate (1% in water), and again air- and vacuum-dried. TEM images were the recorded using a JEM-1011 instrument.

Cell Culture Medium. Roswell Park Memorial Institute (RPMI) medium, Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, fetal bovine serum (FBS), and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Hyclone-Themo Scientific (Logan, UT). Opti-MEM was purchased from Invitrogen-Gibco (Carlsbad, CA).

Cell Culture. All cells (HeLa, MCF7, Huh7, MDAMB-231, MRC5) were cultured in RPMI medium or DMEM supplemented with 10% FBS or FCS, 100 μg/mL of streptomycin, and 100 U/mL of penicillin at 37 °C in a 5% CO_2 incubator. Cells were split, using trypsin/EDTA medium, when almost confluent. All cells were seeded at a density of 2.5 × 10^5 cells/well.

Cellular Uptake Assays. All cells were transfected in the absence of serum with Fl–VEGF–siRNA in the presence of a VB (**VB2**, **VB3**, **VB5**, **VB6**, or **VB7**), lipofectamine 2000 (concentration determined using an Invitrogen kit), or without any transfection reagent. The media were removed after incubation of the cells at 37 °C for 6 h in a CO_2 incubator. The cells were washed with DPBS buffer (1 × 330 μL/well), treated with trypsin (150 μL/well), and incubated for 3 min. DPBS (150 μL/well) was then added to the trypsin solution above, pipetted, and transferred into each well of solution of an e-tube and centrifuged (3000–4000 rpm); the supernatant was removed. DPBS (300 μL/e-tube) was added to the pellets of the cells; each sample was vortexed for 1 min and then centrifuged (3000–4000 rpm); the DPPS was then removed. The same washing procedure was repeated three times. Lysis buffer (70 μL/well) was added and then the samples were vortexed for 20 min at 25 °C and centrifuged (3000–4000 rpm); the supernatant was transferred (50 μL/e-tube) into each well of a 96-well plate (black polystyrene). The absorbance was measured at a wavelength of 485/535 nm using a VICTOR multilabel plate reader (PerkinElmer).

Drug Inhibition Assay. MCF7 cells were preincubated with NaN_3 (0.1%)/2-deoxyglucose (50 mM), genistein (200 mM), or amiloride (200 μg/mL) for 1 h, or with methyl-β-cyclodextrin (5 mM) or chlorpromazine (10 μg/mL) for 15 min, in serum-free MEM at 37 °C in a 5% CO_2 incubator. The cells were washed with DPBS and then the media were changed to fresh media containing the siRNA/**VB7** [siRNA (100 nM)/**VB7**; molar ratio: 1:500] and further incubated for 3 h at 37 °C in a 5% CO_2 incubator. After exposure to siRNA/**VB7** and inhibitors for a desired period of time, the cells were washed with DPBS and then trypsinized and processed for measurement of their fluorescence intensity, as described for the cellular uptake experiments. All inhibitors were obtained from Sigma–Aldrich.

WST-1 Assays. The VBs (molar ratios: 300:1 and 500:1) were complexed with VEGF–siRNA (100 nM) and added to the cells, which were then incubated at 37 °C under 5% CO_2 for 24 h. After incubation, the media were removed, the cells were washed with DPBS buffer (3 × 100 μL/well), and then WST-1 reagent (1 mg/mL of WST-1 dissolved in phenol red free medium, Roche; 100 μL) was added to each well. The mixtures were incubated at 37 °C for 4 h. After incubation, the absorbance was measured at a wavelength of 450 nm, using a UVM 340 microplate reader (ASYS), and converted to the percentage of cell viability (relative to control cells).

VEGF ELISAs. All cells were transfected in the absence of serum with VEGF–siRNA using the VBs or Lipofectamine 2000 (Invitrogen), or in the absence of any transfection reagent. The cells were left to incubate at 37 °C for 6 h in a 5% CO_2 incubator and then washed with DPBS buffer (3 × 330 μL/well), followed by 18 h of incubation with cell medium [DMEM or RPMI medium containing 10% FBS or FCS (330 μL/well)]. The cell medium was collected and analyzed for the VEGF expression level using a QIA51 VEGF ELISA Kit (Human, Calbiochem).

RT-PCR Experiment. VEGF siRNA-treated cells were washed with PBS (phosphate buffered saline) and lyzed in 1.0 mL of TRIzol-reagent (Invitrogen) and total RNA was isolated. A 1 μg sample of RNA was used in reverse transcription with Improm-IITM Reverse Transcription System (Promega) and the procedures were performed by the

manufacturer's protocols. The reverse transcription reaction was carried out at 25 °C (5 min), 42 °C (60 min), and 70 °C for 15 min followed by PCR: 1 cycle, 95 °C, 5 min; 30 cycles, 95 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s; 1 cycle, 72 °C, 5 min. For VEGF mRNA amplification, forward and reverse primers were 5′-atgaactttctgctgtcttgggt-3′ and 5′-tcaccgcctcggcttgtcaca-3′, respectively. And, for the control mRNA of GAPDH, forward and reverse primers were 5′-gagtcaacggatttggtcgt-3′ and 5′-ttgattttggagggatctcg-3′, respectively. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel (100 V, 30 min) stained with ethidium bromide.

***Ex Vivo* Imaging.** MDA-MB 231 cells (5×10^6 cells/mouse) were injected subcutaneously into the right flank of female BALB/c nu/nu mice. Two days after the injection, each mouse was randomly placed in one of two groups: a group treated with Fl−VEGF−siRNA only or a group treated with siRNA/**VB7** (siRNA: 0.1 mg/kg; molar ratio: 1:500). For visualization, siRNA was conjugated with 5,6-FAM, a derivative of fluorescein. siRNA (0.1 mg/kg) or siRNA with a biotin-derived compound was injected into the tail vein. After 1 h, each mouse were sacrificed through cervical dislocation, and its organs extracted. Fluorescence images were recorded within a minute using IVIS (PerkinElmer, USA).

All the mouse experiments were performed in the POSTECH animal facility and were approved by the POSTECH Institutional Animal Care and Use Committee (approval number: 2014-03-0002).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures; spectral charecterization data of VBs; chemical structures of precursors for the VBs; ^{1}H and ^{13}C NMR spectra; TEM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

N/P ratio, no. of amino group (cation) from **VB**/no. of phosphate group (anions) from siRNA; Boc, *tert*-butyloxycarbonyl; THF, tetrahydrofuran; *t*-BuOH, tertiary butylalcohol; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay

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