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Environment-Responsive Polymers for Coating of Pharmaceutical Nanocarriers^{1,2}

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Abstract

Polyethylene glycol derivatives, such as block copolymers of polyethylene glycol and diacyllipids (for example, phosphatidylethanolamine) are widely used for surface modification of various pharmaceutical carriers in order to impart them longevity in the body. To make polyethylene glycol detachable from the surface of pharmaceutical carrier and facilitate the interaction of the carrier with target cells when in pathological zone, we have prepared a set of polyethylene glycol–phosphatidylethanolamine block copolymers with the pH sensitive hydrazone bond between polyethylene glycol and phosphatidylethanolamine, which destabilizes at lowered pH values typical for tumors and inflammation zones. We have demonstrated that the stability of the hydrazone bond at normal physiological pH (7.4) as well as the rate of its hydrolysis at pH 6 and below strongly depend on the type of substitutions at this bond. Using aliphatic and aromatic aldehydes and ketones, polyethylene glycol–phosphatidylethanolamine block copolymers were prepared with different stabilities and degradation rates, which can be useful in constructing stimuli-sensitive pharmaceutical carriers.

INTRODUCTIONS

The development of effective drug delivery systems remains an important task of pharmaceutical sciences [1]. The ultimate goal in drug delivery is to achieve therapeutic concentrations of the drug at the target site while drug concentrations in other tissues are kept in safe levels. This has a particular importance in case of cancer treatment, where the challenge is to selectively destroy the tumor without damaging normal tissues. The issue however, remains, how to achieve fast and effective drug release from the pharmaceutical carrier (liposome) when it has already reached its target [2-4]. This issue is equally important when long-circulating polyethylene glycol (PEG) containing drug delivery systems are used [5-7], since PEG prevents normal interaction of the carrier with cells, or when drug carrier is intended for the intracellular penetration [8, 9] and a properly scheduled cytoplasmic release of the active drug is expected to prevent its lysosomal degradation [10].

There are several approaches to solve this problem including the use of stimuli-sensitive pharmaceutical nanocarriers, which is based on the fact that many pathological sites including tumors demonstrate hyperthermia or acidosis [11-13]. In general, environmentally-sensitive carriers exhibit dramatic changes in their swelling behavior,

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permeability, or stability in response to changes in the pH, or ionic strength, or temperature of the surrounding media [14].

Such systems are frequently based on the use of various stimuli-sensitive polymers attached onto or incorporated into pharmaceutical carriers. Thus, for example, accelerated doxorubicin (DOX) release was observed from DOX-loaded poly(β -benzyl-L-aspartate) copolymer micelles after lowering the surrounding pH from 7.4 to 5.0, suggesting a pH-sensitive release of DOX from the micelles [15]. Hydrophobically-modified copolymers of *N*-isopropylacrylamide bearing a pH-sensitive moiety were investigated for the preparation of pH-responsive liposomes and polymeric micelles [16]. The copolymers having the hydrophobic anchor randomly distributed along the polymeric chain were found to more efficiently destabilize egg phosphatidylcholine/cholesterol (**EPC/Ch**) liposomes than the alkyl-terminated polymers. Release various markers as well as real drugs from copolymer-modified liposomes was shown to be pH-dependent. pH-Responsive polymeric micelles loaded with the photosensitizer aluminum chloride phthalocyanine were found to exhibit increased cytotoxicity against EMT-6 mouse mammary cells in vitro than the control Cremophor EL formulation [17, 18]. The authors [19, 20] have formulated pH-sensitive polymeric mixed micelles composed of poly(L-histidine) (polyHis; $M_w = 5000$)/PEG ($M_n = 2000$) and poly(L-lactic acid) (PLLA; $M_n = 3000$)/PEG ($M_n = 2000$) block copolymers with or without folate conjugation. The polyHis/PEG micelles showed accelerated adriamycin release as the pH decreased from 8.0. Depending on the amount of PLLA/PEG, the mixed micelles were destabilized in the pH range of 7.2–6.6 (triggering pH for adriamycin release). When the mixed micelles were conjugated with folic acid, the in vitro results demonstrated that the micelles were more effective in tumor cell killing due to accelerated drug release and folate receptor-mediated tumor uptake.

Certain pH-sensitive linkages have been popularly used to allow the drug release, protective “coat” removal, or new function appearance because of their fast degradation in acidified pathological sites [21–23]. These include cis-aconityls [24, 25], electronrich trityls [26], polyketals [27], acetals [28, 29], vinyl ethers [30, 31], hydrazones [32–34], poly(orthoesters) [35], and thiopropionates [36]. Such constructs may turn out to be useful for the site-specific delivery of drugs at the tumor sites [12], infarcts [37], inflammation zones [38] or cell cytoplasm or endosomes [39], since at these “acidic” sites, pH drops from the normal physiologic value of pH 7.4 to pH 6.0 and below.

Thus, different acetal-based conjugates have been synthesized using model drugs and PEG [40]. The hydrolysis kinetics of the conjugates had half-lives ranging from less than 1 min to several days at pH 5.0, with slower hydrolysis at pH 7.4 in all cases. Encrypted polymers containing pH-sensitive acetal linkage between either oligonucleotide or macromolecule and PEG showed direct vesicular escape and efficiently deliver oligonucleotides and macromolecules into the cytoplasm of hepatocytes [41]. Acetal-based acid-degradable protein-loaded microgels also have showed promising results for delivery of protein-based vaccines [42]. Acid-labile PEG [43] conjugated vinyl ether lipids were synthesized and used at low molar ratios to stabilize the nonlamellar, highly fusogenic lipid, dioleoylphosphatidyl ethanolamine, as unilamellar liposomes [31]. Acid-catalyzed hydrolysis of the vinyl ether bond destabilized these liposomes by removal of the sterically-stabilizing PEG layer, thereby promoting contents release on the hours timescale at pH < 5.

It was also reported that hydrazone-based polymer–daunorubicin conjugates have better cytotoxicity than the analogues containing non-cleavable linkers between those conjugates [44]. In another study, the micelles formed by amphiphilic AB and ABA block copolymers from poly(ortho esters) and PEG were stable in PBS at pH 7.4 and 37°C for 3 days and in a citrate buffer at pH 5.5 and 37°C for 2 h [35]. The remarkably enhanced gene silencing in

hepatoma cells was achieved by assembling lactosylated-PEG-siRNA conjugates bearing acid-labile beta-thiopropionate linkages into polyion complex (PIC) micelles through the mixing with poly(L-lysine) [45]. Almost 100-fold enhancement in gene silencing was achieved for the micelle system compared to that of the free conjugate. Kataoka's group [46] also architected three types of newly engineered block copolymers forming polyplex micelles useful for oligonucleotides and siRNA delivery: (1) PEG-polycation diblock copolymers possessing diamine side-chain with distinctive pKa for siRNA encapsulation into polyplex micelles with high endosomal escaping ability, (2) lactosylated PEG-(oligonucleotide or siRNA) conjugate through acid-labile beta-thiopropionate linkage to construct pH-sensitive PIC micelles, and (3) PEG-poly(methacrylic acid) block copolymer for the construction of organic/inorganic hybrid nanoparticles encapsulating siRNA.

Earlier, we have demonstrated the utility of pH-sensitive hydrazone bond-based PEG – phosphatidylethanolamine (PE) conjugates in preparing double-targeted stimuli-sensitive pharmaceutical nanocarriers [47, 48]. Two important temporal characteristics of such carriers include their sufficiently long lifetime (few hours) under normal physiological conditions and their sufficiently fast (under 30 min) destabilization within the acidic target. Since real practical tasks may require different times for such carriers to stay in the blood and to release their contents (or “develop” an additional function) inside the target, we have synthesized a series of PEG-PE conjugates with different substituents at the hydrazone bond (PEG-HZ-PE) and evaluated their hydrolytic stability at normal and slightly acidic pH values. These conjugates differed from each other with respect to the exact structure of groups forming the hydrazone linkage between phospholipid and PEG. The characterization of the *in vitro* behavior of these conjugates has provided important information useful for future design and development of pH-sensitive nanocarriers with controlled properties.

MATERIALS

EPC; Ch; 1,2-dioleoyl-3-trimethylammonium propane; phosphatidylthioethanolamine, PE-SH; 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, DOPE; 1,2-dipalmitoyl-*sn*-glycero-3-phosphothioethanolamine (sodium salt), DPPE-SH; 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt), PE-Rh, were purchased from Avanti Polar Lipids, (Alabaster, AL). Methoxy polyethylene glycol butyraldehyde ($M_w = 2000$, mPEG₂₀₀₀-butyraldehyde), and mPEG₂₀₀₀-SH were purchased from Nektar Therapeutics (Huntsville, AL); maleimide-PEG₁₀₀₀-NHS was from Quanta Biodesign (Powell, OH). Succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate, SMCC, was purchased from Pierce Biotechnology Inc. (Rockford, IL); 4-succinimidyl formylbenzoate, SFB, was purchased from Molecular Biosciences (Boulder, CO); 4-acetyl phenyl maleimide, was purchased from Sigma-Aldrich. The following linkers have been used in this study: (*N*-ε-maleimidocaproic acid) hydrazide, EMCH; 4-(4-*N*-maleimidophenyl) butyric acid hydrazide hydrochloride, MPBH; and *N*-(k-maleimoundecanoic acid) hydrazide, KMUH from Pierce; 2-acetamido-4-mecrcaptobutanoic acid hydrazide, AMBH, from Molecular Probes (Carlsbad, CA); and succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate hydrazide, SMCCH, from Molecular Biosciences.

Triethylamine was purchased from Aldrich Chemicals; TATp-cysteine—from Research Genetics (Huntsville, AL). All solvents were purchased from Fisher Scientific (HPLC grade) and used without further purification.

Lewis Lung Carcinoma (LLC) cell line was purchased from ATCC (Rockville, MD). Delbecco's minimal essential medium, complete serum free medium and fetal bovine serum were purchased from Cellgro (Kansas City, MO).

All reactions were monitored by TLC using 0.25 mm × 7.5 cm silica plates with UV-indicator (Merck 60F-254), and mobile phase chloroform:methanol (80 : 20% v/v). Phospholipid and PEG alone or their conjugates were visualized by phosphomolybdic acid and Dragendorff spray reagents. Silica gel (240–360 μm) and size exclusion media, Sepharose CL4B (40–165 μm) and Sephadex G25m (Sigma-Aldrich) were used for silica column chromatography and size exclusion chromatography, respectively.

METHODS

Synthesis of Hydrazone-based PEG–HZ–PE Conjugates [48, 49]

Synthesis of aliphatic aldehyde-derived hydrazone-based PEG–HZ–PE

conjugates—On the first step 22 μmoles of PE were mixed with 1.5 molar excess of each acyl hydrazide linker (Table 1) in 3 ml anhydrous methanol containing 5 molar excess of triethylamine over lipid. The reaction was performed at 25°C under argon for 8 h. Solvent was removed under reduced pressure, and the residue was dissolved in chloroform and applied to a 5-ml silica gel column which had been activated (150°C overnight) and pre-washed with 20 ml of chloroform. The column was equilibrated with an additional 15 ml of chloroform followed by 5 ml of each of the following chloroform:methanol mixtures 4 : 0.25, 4 : 0.5, 4 : 0.75, 4 : 1, 4:2 and, finally, with 6 ml of 4 : 3 v/v. The phosphate-containing fractions eluting in 4 : 1, 4 : 2 and 4 : 3 chloroform:methanol (v/v) were pooled and concentrated under reduced pressure. The product was stored in glass ampoules as chloroform solution under argon at –80°C.

For the activation of phospholipid with AMBH, a maleimide derivative of PE was prepared using SMCC. In brief, PE, in chloroform was reacted with 1.5 molar excess of SMCC over lipid in presence of 5 molar excess of triethylamine under argon for 5 h. The maleimide-derivative was separated from excess SMCC on silica gel column using chloroform:methanol (4 : 0.2 v/v) mobile phase. The elution fractions containing Ninhydrin-negative and phosphorus-positive fractions were pooled and concentrated under reduced pressure. DOPE-maleimide was further used to synthesize AMBH-activated derivative of phospholipid, by reacting with 1.5 molar excess of AMBH using triethylamine as catalyst.

On the second step 2 (schemes 1 and 2), 21 μmoles of mPEG₂₀₀₀-butyraldehyde were reacted with 14 μmoles of linker-activated phospholipid in 2 ml chloroform at 25°C in a tightly closed reaction vessel. After an overnight stirring, chloroform was evaporated under vacuum in rotary evaporator. The excess mPEG₂₀₀₀-butyraldehyde was separated from PEG–HZ–PE conjugates using gel filtration chromatography. The gel filtration chromatography was performed using Sepharose-CL4B in pH 9–10 degassed ultra pure water (elution medium) in 1.5 × 30 cm glass column. The thin film formed in round bottom flask after evaporating chloroform was hydrated with the elution medium and applied to the column. The micelles formed by PEG–HZ–PE conjugate were the first to elute from the column. Micelle containing fractions were identified by Dragendorff spray reagent and pooled together, kept in freezer at –80°C overnight before subjecting to freeze drying. The freeze dried PEG–HZ–PE conjugates were weighed and stored at –80°C as chloroform solution.

Synthesis of aromatic aldehyde-derived hydrazone-based PEG–HZ–PE

conjugates—Firstly, 35 μmoles of PE in chloroform were mixed with 2 molar excess of SFB in the presence of 3 molar excess triethylamine over lipid. After stirring for 3 h, solvent was evaporated, residue was redissolved in chloroform and product was separated on silica gel column using acetonitrile:methanol mobile phases: 4 : 0, 4 : 0.25, 4 : 0.5, 4 : 0.75 and 4 : 1 v/v. The fractions containing product were identified by TLC analysis, pooled and concentrated. The product was stored as chloroform solution at –80°C.

On the second step (scheme 3) 40 μ moles of mPEG₂₀₀₀-SH in chloroform were mixed with two molar excess of acyl hydrazide cross-linkers: EMCH, MPBH, and KMUH in the presence of 5 molar excess of triethylamine over lipid. The excess of EMCH was separated from the product by SEC using Sephadex G25m media. The acyl hydrazide derivatives of PEG, were freeze dried and stored as chloroform solution at -80°C .

On the last step (scheme 4) 1.5 molar excess of SFB activated phospholipid, was reacted with acyl hydrazide-derivatized PEGs in chloroform at room temperature. After overnight stirring, chloroform was evaporated under reduced pressure. The PEG-HZ-PE conjugate was purified by SEC using Sepharose CL4B as described before.

Synthesis of aromatic ketone-derived hydrazone-based mPEG-HZ-PE conjugates (scheme 5)—mPEG₂₀₀₀-SH, was reacted with 2 molar excess of SMCCH, in presence of triethylamine for 8 h in dry chloroform. Chloroform was evaporated, and the residue was dissolved in water. The PEG-hydrazide derivative, was separated and purified by SEC using Sephadex G25m media. The product was freeze dried and stored as chloroform solution at -80°C .

After that 40 μ moles of 4-acetyl phenyl maleimide were reacted with 27 μ moles of PE-SH, in the presence of triethylamine overnight with constant stirring under inert atmosphere of argon. The product, was separated on a silica gel column using chloroform:methanol mobile phase (4 : 1 v/v). The fractions containing product were identified by TLC, pooled and concentrated. Aromatic ketone-activated phospholipid was stored as chloroform solution at -80°C .

On the last step hydrazide-activated PEG derivative, was reacted with 1.5 molar excess of the aromatic ketone-derivatized phospholipid, overnight under the constant stirring at room temperature. The PEG-HZ-PE conjugate, was separated and purified by SEC using Sepharose-CL4B.

In vitro pH-dependent Degradation of PEG-HZ-PE Conjugates

The time-dependant degradation of PEG-HZ-PE micelles incubated in buffer solutions (phosphate buffer saline, pH 7.4 and pH 5.0) maintained at 37°C was followed by HPLC using Shodex KW-804 size exclusion column with the elution phosphate buffer (100 mM phosphate, 150 mM sodium sulfate at pH 7.0) run at 1.0 ml/min. For fluorescent detection (Ex 550nm/Em 590nm) of micelle peak, PE-Rh (1 mol % of PEG-PE) was added to the PEG-PE conjugate in chloroform. A film was prepared by evaporating the chloroform under argon stream and hydrated with the phosphate buffer saline, pH 7.4 or 5.0 (adjusted by pre-calculated quantity of 1 N HCl). A peak that represents micelle population appeared at the retention time between 9–10 min. The degradation kinetics of micelles was assessed by following the area under micelle curve.

RESULTS AND DISCUSSION

Synthesis of Hydrazone-Bbased PEG-HZ-PE Conjugates

The success of hydrazones as pH-sensitive linkages derives from the fact that their hydrolytic stability is governed by the nature of hydrazone bond formed. Hydrazones are much more stable than imines as a result of the delocalization of the π -electrons in the former. In fact, parent hydrazones are too stable for the application in drug delivery systems, and an electron withdrawing group has to be introduced to moderate the stability by somewhat disfavoring electron delocalization throughout the molecule as compared to the parent hydrazone. Hydrazones can be prepared from aldehydes or ketones and hydrazides under very mild conditions including aqueous solutions. Hydrazone bond formation can take

place even *in vivo* from separate fragments which self-assemble under physiological conditions [50].

We have applied different synthetic methods based on the use of various aldehydes that can produce the hydrazone linkage between PEG and PE [48]. Synthesis of aliphatic aldehyde-derived hydrazone containing PEG–PE conjugate was pursued in two steps. First, phospholipid was activated with four different acyl hydrazides. The sulfhydryl reactive group of PE-SH was reacted with maleimide end of maleimido acyl hydrazides through the Michael addition, thus providing acyl hydrazide activated PE. mPEG₂₀₀₀-butyraldehyde, an aliphatic aldehyde, was then reacted with acyl hydrazide activated PE to get hydrazone based PEG–PE conjugate.

To synthesize aromatic aldehyde-derived hydrazone, an aromatic aldehyde moiety was introduced into the phospholipid by reacting SFB with PE under mild alkaline conditions. The acyl hydrazide-PEG derivatives were synthesized using mPEG₂₀₀₀-SH and maleimido acyl hydrazides (EMCH, MPBH, and KMUH). The SFB-activated phospholipid was then reacted with acyl hydrazide derivatized PEG. Aromatic ketone-derived hydrazone-based PEG–PE conjugates were synthesized by reacting aromatic ketone-activated phospholipids with acyl hydrazide-activated PEG [49].

In vitro pH-dependent Degradation of PEG–HZ–PE Conjugates

All PEG–HZ–PE conjugates spontaneously form micelle in aqueous media [51]. The stability of hydrazone-based PEG–PE conjugates incubated at physiological pH 7.4 and acidic pH 5.0 in buffer solutions maintained at 37°C was investigated by HPLC. For this purpose, the area under the micelle peak of PEG–HZ–PE (R_t 9–10 min) was observed over a period of time. PEG–HZ–PE conjugates derived from an aliphatic aldehyde and different acyl hydrazides were found to be highly unstable under acidic conditions, with the micelle peak was completely disappearing within 2 min incubation at pH 5.0. At the same time, these conjugates were relatively stable at physiological pH: the PEG–HZ–PE conjugate, with AMBH as cross-linker showed the half-life of 150 min followed by EMCH (120 min), MPBH (90 min), and KMUH (20 min) (Table 1). The rate of hydrolysis among the aliphatic aldehyde-derived hydrazone-based PEG–PE conjugates at pH 7.4 seems to be dependent on carbon chain length of acyl hydrazide. The increase in number of carbon atoms in acyl hydrazide led to increase in rate of hydrolysis: PEG–PE conjugate, acyl hydrazide with ten C atoms (KMUH) > acyl hydrazide with five C atoms (EMCH) > acyl hydrazide with three C atoms (AMBH). Introducing an aromatic character within the carbon chain of acyl hydrazide led to an increase in hydrolysis as observed in case of MPBH and EMCH linkers (rate of hydrolysis of MPBH > EMCH).

Alternatively, the PEG–HZ–PE conjugates derived from an aromatic aldehyde and acyl hydrazides were found to be highly stable at pH 7.4 and 5.0 (Table 1). The half-life values were not attained at either of those pH values even at the end of incubation period of 72 h at pH 7.4 and 48 h at pH 5.0 buffer solutions maintained at 37°C. The resistance to hydrolysis exhibited by hydrazones derived from aromatic aldehydes can be attributed to the conjugation of the π bonds of –C=N bond of the hydrazone with the π bonding benzene ring. Thus, it supports the finding that hydrazones formed from aromatic aldehydes are more stable to acidic hydrolysis than those formed from aliphatic ones [52, 53]. The hydrazone hydrolysis involves the protonation of the –C=N nitrogen followed by the nucleophilic attack of water and cleavage of C–N bond of tetrahydrazone intermediate [54]. Any of these steps is determining and dependant on the pH. The substituents on the carbonyl reaction partner influence the rate of hydrolysis through altering the pK_a of the hydrazone with electron donating substituents facilitating protonation of the –C=N nitrogen [55].

This would support the fact that PEG–HZ–PE conjugates containing hydrazone bond derived from the aliphatic aldehyde are more prone to hydrolytic degradation. Aromatic aldehyde-derived hydrazone bond is too stable for the purpose of pH-triggered drug release. Careful selection of an aldehyde and an acyl hydrazide would be necessary for the application of the hydrazone-based chemistry for the development of pH-sensitive pharmaceutical nanocarriers.

As scheme 5 shows, an aromatic ketone-derived hydrazone bond was introduced between PEG and PE. The presence of a methyl group (electron-donating) on the carbonyl functional group would provide a sufficient lability of the hydrazone bond under mildly acidic conditions while an immediate aromatic ring (electron-withdrawing) next to the hydrazone bond would offer the stability under acidic and neutral conditions. mPEG–HZ–PE conjugate, wherein the hydrazone bond is derived from an aromatic ketone, exhibited the half-lives of 2–3 h at slightly acidic pH values, and much higher stability (up to 40 h) at the physiological pH (Table 1).

CONCLUSIONS

pH-Sensitive PEG–HZ–PE conjugates based on hydrazone bond chemistry were synthesized. The pH-dependant hydrolytic kinetics could be tuned using appropriate aldehyde or ketone and acyl hydrazide. These conjugates could have various applications in targeted drug delivery systems, e.g., the development of the targeted drug carriers carrying the temporarily hidden function and a detachable PEG–HZ–PE, which, in addition to prolonging circulation half-life of carriers, can expose the hidden function only under the action of certain local stimuli (such as lowered pH).

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Scheme 2.
Synthesis of PEG-HZ-PE conjugate using AMBH-activated phospholipid.

Scheme 3.
Synthesis of acyl hydrazide-activated PEG.

Scheme 4.
Synthesis of PEG-HZ-PE conjugate.

Scheme 5.

Synthesis of aromatic ketone-derived hydrazone-based mPEG-HZ-PE.

Table 1

Half-lives of different hydrazone-based PEG–HZ–PE conjugates incubated in phosphate buffered saline, at pH 7.4 and at pH 5.0 at 37°C

PEG–HZ–PE conjugate prepared via the following cross-links	Half-life (h)	
	pH 7.4	pH 5.0
Aliphatic aldehyde		
EMCH	2.0	<0.03
MBPH	1.5	<0.03
KMUH	0.33	<0.03
AMBH	2.5	<0.03
Aromatic aldehyde		
EMCH	>72	>48
MBPH	>72	>48
KMUH	>72	>48
SMCCH	40	2.0