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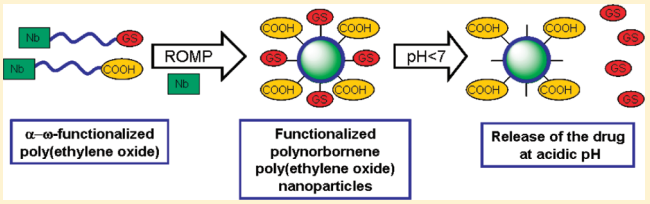
Synthesis of pH-Sensitive Particles for Local Delivery of an Antibiotic via Dispersion ROMP

Loïc Pichavant,[†,‡] Chantal Bourget,[‡] Marie-Christine Durrieu,[‡] and Valérie Héroguez*[,†]

[†]CNRS UMR5629, Laboratoire de Chimie des Polymères Organiques, IPB-ENSCBP, Université de Bordeaux 1, 16 avenue Pey Berland, F-33607 Pessac, France

[‡]INSERM, Bioingénierie tissulaire, U1026, Université de Bordeaux Segalen, 146 rue Léo Saignat F-33000 Bordeaux, France

ABSTRACT: The synthesis and the *in vitro* antibacterial activity of pH-sensitive functionalized nanoparticles for the local delivery of antibiotic are described. Nanoparticles (NPs) are formed by ring-opening metathesis copolymerization (ROMP) in dispersed medium of norbornene (Nb) with α,ω-functionalized macromonomers. Macromonomers are ended with gentamicin sulfate (GS) or carboxylic acid groups. GS, linked thanks to a pH-sensitive bond, can be released in acidic medium whereas acidic function allows the future covalent grafting of these NPs onto biomaterial surface in order to synthesize innovating GS-loaded biomaterials. In this paper, we focused on the synthesis and the characterization of biofunctional NPs and their *in vitro* antibacterial activities. NPs have been synthesized with a controlled rate of GS and their stability in aqueous medium has been proved. Minimum inhibitory concentration (MIC) measurements using *Staphylococcus epidermidis* as bacterial strain after the GS release at acidic pH have permitted us to prove the efficiency of this system.



1. INTRODUCTION

Polymeric nanoparticles have been greatly used in the past decades as drug carriers, allowing a more efficient delivery of active molecules at a targeted site, increasing in this way the specific activity and reducing side effects.[1] These nanoparticles are usually formed by self-assembly of block copolymer with low critical micelle concentration encapsulating drugs, or by the formation of reticulated polymer matrix in which drugs are dissolved or dispersed and released by diffusion.[2] Nevertheless, these kinds of systems do not allow a local controlled release at the target site.

In the last ten years, new generations of therapeutic nanoparticles (NPs) sensitive to environmental changes such as pH variation and involving a more specific drug release have been studied.[3] These NPs have essentially been designed to treat human tumors which exhibit acidic microenvironments,[4−6] but could equally been employed to treat bacterial infections. Different mechanisms are presented in the literature involving the release of drug from polymer NPs through a pH variation. The swelling or dissolution of self-assembled NPs based on pH decrease permit this release.[7−12] In some studies, NPs are built with pH-sensible links and hydrolyzed at acidic pH.[13−16] pH hydrolyzable functions are multiple[17−19] and their choice depend on the polymer structure and the pH value.[3] Among them, the imine function—or Schiff base—is usually employed because of its high sensibility of the pH variation.[20,21] For such systems, the drug release usually implies a destructuration of the nano-objects and the presence of degradation products in the organism.

Our aim, in this paper is to focus on another innovating way and to demonstrate the feasibility of biofunctional NPs synthesis able to release an antibiotic, gentamicin, able to fight infection. The human immune system is notable for its ability to combat infectious microorganism by eliciting inflammatory responses.[22] Acute phase proteins such as C-reactive protein (CRP)[23] and cytokines[24] are elevated markedly in association with infection and inflammation. During this process, local acidosis occurs due to massive infiltration of neutrophils and macrophages[25] to the site of infection, These pathological conditions can decrease the pH to as low as 5.5−7.0.[11]

Herein, we report on a route, which has never been explored, to synthesize pH-sensible drug delivery devices based on the ring-opening metathesis copolymerization (ROMP) of norbornene with a pH-sensitive α-norbornenyl-poly(ethylene oxide) macromonomer. ROMP is an elegant and well-established way for the formation of monodispersed NPs with hydrophobic polynorbornene core and hydrophilic poly(ethylene oxide) shell.[26,27] In this work, we explore the macromonomer route to plurifunctionalize nanoparticles with both prodrugs and reactive chemical groups as carboxylic acid (Figure 1). The benefits of such strategy compared with the other drug delivery systems are multiple. First, it allows a plurifunctionalization with two—or more—drugs: a great advantage in the case of a bacterial infection to avoid antibiotic resistance.[28] Second, carboxylic acid groups could permit the anchoring of the NPs on biomaterial surface and the local delivery of the drug on the site of infection. Finally, it allows obtaining NPs bearing high drug densities

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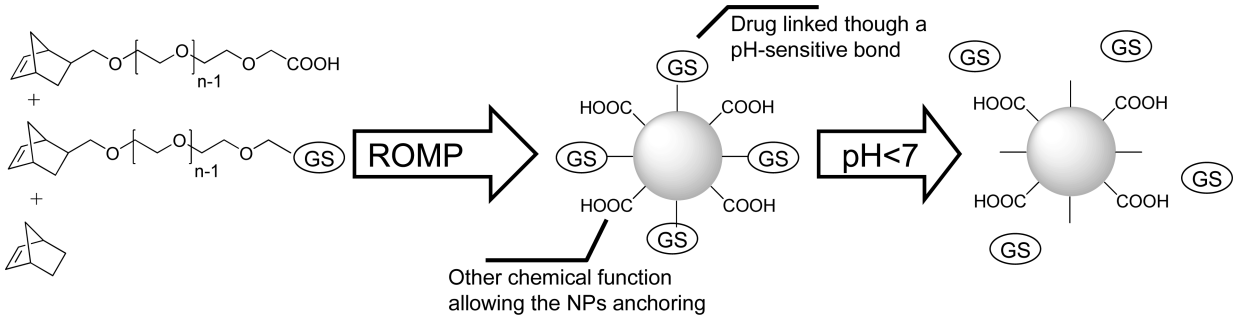


Figure 1. Functionalized NPs formation and GS release at acidic pH.

(ca. 0.3 mmol. of drug per gram of NPs). This density is perfectly controlled and can be modulated as a function of the macromonomer ratio. In this work, the drug chosen is the gentamicin sulfate (GS), a wide-spectrum antibiotic usually used to prevent infections in orthopedic surgery. Because of its three amine groups, GS can easily be linked on ω-modified macromonomers through a pH-sensitive imine bond. Polynorbornene is commonly used for biological applications because of its noncytotoxicity.[29−31]

This paper will be divided into three parts: In a first part, we have drawn particular attention to the synthesis and the characterization of the α,ω-functionalized poly(ethylene oxide) macromonomers. Then the NPs formation by ROMP in dispersed medium has been described and the influence of the functionalization on their colloidal stabilities has been discussed. Finally, we have chosen to prove the efficacy of the GS release at acidic pH by using the antibacterial properties of this molecule. Quantitative bacterial inhibition tests—minimum inhibitory concentration (MIC) measurements—have been carried out after the drug release at acidic pH and during different durations using *Staphylococcus epidermidis* as bacterial strain.

2. EXPERIMENTAL PART

Material. Ethylene oxide (EO; 99.5%; Aldrich) was stirred over sodium at −30 °C for 2 h and subsequently cryodistilled. Tetrahydrofuran (THF; J.T. Baker) was cryodistilled from sodium benzophenone before use. Ethanol (96%; purissimum grade pur; Xilab) and dichloromethane (purissimum grade pur, Xilab) were degassed before use. Diphenyl methyl potassium (DPMK; 0.61 mol·L^{-1} in THF) was synthesized and dosed according to well-established procedures. Sodium hydride (60% in dispersion in mineral oil; Aldrich) was washed with anhydrous heptane before use. Grubbs first generation complex Cl_2-$(PCy_3)_2Ru{=}CH{-}Ph$ (Aldrich; stored in a glovebox under Argon atmosphere) was used as reserved. Norbornene (99% (GC); Aldrich), 5-norbornene-2-methanol (98%; mixture of endo and exo; Aldrich), 2-bromoacetic acid (99%; ACROS Organics), 2-bromoethyl acetate (97%; Aldrich), bromoacetaldehyde diethyl acetal (97%; Aldrich), and gentamicin sulfate (Tecknimed) were used without further purification.

Methods. Polymerizations of ethylene oxide are performed under anhydrous inert atmosphere. ROMP is performed in a glovebox. ^{1}H NMR spectra are obtained using a Bruker spectrometer 400 MHz in $CDCl_3$ or D_2O used as solvent. Size exclusion chromatography analyses are carried out on a Varian apparatus equipped with TOSOHAAS TSK gel columns and a refractive index detector. THF is used as the solvent at a flow rate of 1 mL·min^{-1}. Mass calibration is achieved with narrow polydispersity poly(ethylene oxide) standards. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI−TOF MS) spectra are performed on a Voyager mass spectrometer (Applied Biosystems). The instrument is equipped with a pulsed N_2 laser (337 nm) and a time-delayed extracted ion source. Spectra are recorded in the positive-ion mode using the reflectron and with an accelerating voltage of 20 kV. 3-Indoleacetic acid (IAA) is used as matrix. Dithranol is used as non acidic matrix for GS-functionalized macromonomer having a pH-sensitive bond. For ROMP in dispersion, conversions of norbornene (Nb) are determined by gas chromatography with a trace of dodecane as internal standard, using a VARIAN GC3900 (GC retention times: t^{GC}_{Nb} = 1.77 min; $t^{GC}_{dodecane}$ = 8.55 min), while the macromonomer conversions are followed by SEC (SEC retention times: $t^{SEC}_{macromonomers}$ = 18.75 min; $t^{SEC}_{dodecane}$ = 31.70 min). Dynamic light scattering (DLS) measurements are performed using a MALVERN zetasizer Nano ZS equipped with He−Ne laser (4 mW; 633 nm). Before measurements, latexes are diluted about 800 times to minimize multiple scatterings caused by high concentration. The scattering angle used is 173°. Thin-layer chromatography is carried out using aluminum oxide as stationary phase and an ethyl acetate/methanol (4:3) mixture as mobile phase. $KMnO_4$ (0.02 mol·L^{-1}) has been used as developer to oxidize the insaturations of the norbornenyl entities and ninhydrine (3 g·L^{-1} in ethanol) to prove the presence of amine groups.

Synthesis of α-Norbornenyl Poly(ethylene oxide). α-Norbornenyl poly(ethylene oxide) (**1**) is prepared by anionic ring-opening polymerization of ethylene oxide.[26] First, 0.8 mL of norbornene methanol (0.015 equiv; 6.63 × 10^{-3} mol) is dissolved in 200 mL of THF previously cryodistilled. Then 8.7 mL of DPMK (0.012 equiv; 0.61 mol·L^{-1}) is added. Then 22.5 mL (1 equiv; 0.451 mol) of ethylene oxide stirred over sodium and cryodistilled is promptly added. The mixture is stirred during 48 h under inert atmosphere at room temperature, and the anionic active centers are neutralized with 5 mL of acidic methanol. The polymer is precipitated in anhydrous diethyl ether, filtered, dissolved in dichloromethane, dried with $MgSO_4$, filtered on Celite, concentrated, precipitated in diethyl ether, filtered and dried under vacuum.

83% yield.

^{1}H NMR data in $CDCl_3$: δ (ppm) 1.08−1.79 (m, 4H, $-CH_2-_{cycle}$); 2.72−3.45 (m, 3H, $-CH-_{cycle}$); 3.63 (m, 330H, $-CH_2-O-$); 5.91−6.09 (m, 2H, $-CH{=}CH-_{cycle}$).

Synthesis of α-Norbornenyl-ω-ester Poly(ethylene oxide). α-Norbornenyl-ω-ester poly(ethylene oxide) (**2**) is obtained by nucleophilic substitution of **1** on 2-bromoethyl acetate activated by sodium hydride. Then, 582 mg of washed NaH (10 equiv; 2.42 × 10^{-2} mol) is dissolved in 5 mL of THF. First, 5 g (1 equiv; 1.35 × 10^{-3} mol) of **1** dissolved in 25 mL of THF is added dropwise. The mixture is left for 30 min under stirring. Then 0.68 mL of 2-bromoethyl acetate (4.5 equiv; 6.07 × 10^{-3} mol) is added dropwise. As the mixture is stirred during 12 h at 60 °C, NaH is neutralized with 15 mL of HCl 1 M. The solvent is evaporated. The polymer is dissolved in dichloromethane, dried with $MgSO_4$, filtered, concentrated, precipitated in diethyl ether, filtered, and dried under vacuum.

86% yield; 85% functionalization.

^{1}H NMR data in $CDCl_3$: δ (ppm) 1.27 (t, J = 7 Hz, 2.6H, CH_3-); 1.08−1.79 (m, 4H, $-CH_2-_{cycle}$); 2.72−3.45 (m, 3H, $-CH-_{cycle}$); 3.63 (m, 410H, $-CH_2-O-$); 4.13 (s, 1.7H, $-O-CH_2-COOEt$); 4.21 (q, J = 7 Hz, 2.6H, $O-CH_2-CH_3$); 5.91−6.09 (m, 2H, $-CH=CH-_{cycle}$).

Synthesis of α-Norbornenyl-ω-carboxylic Acid Poly(ethylene oxide). α-Norbornenyl-ω-carboxylic acid poly(ethylene oxide) (**3**) is prepared by two ways:

Route 1. **3** is obtained by nucleophilic substitution of **1** on 2-bromoacetic acid activated by sodium hydride. First, 582 mg of washed NaH (10 equiv; 2.42×10^{-2} mol) is dissolved in 5 mL of THF. Then 5 g (1 equiv; 1.35×10^{-3} mol) of **1** dissolved in 25 mL of THF is added dropwise. The mixture is left to stir for 30 min. Then 105 mg of 2-bromoacetic acid dissolved in 5 mL of THF (4.5 equiv; 6.07×10^{-3} mol) is added dropwise. Stirring the mixture during 12 h at 60 °C, NaH is neutralized with 15 mL of HCl 1M. The solvent is evaporated. The polymer is dissolved in dichloromethane, dried with $MgSO_4$, filtered, concentrated, precipitated in diethyl ether, filtered, and dried under vacuum.

87% yield; 15% functionalization.

^{1}H NMR data in $CDCl_3$: δ (ppm) 1.08−1.79 (m, 4H, $-CH_2-_{cycle}$); 2.72−3.45 (m, 3H, $-CH-_{cycle}$); 3.63 (m, 430H, $-CH_2-O-$); 4.08 (s, 0.2H, $-O-CH_2-COOH$); 5.91−6.09 (m, 2H, $-CH=CH-_{cycle}$).

Route 2: **3** is obtained by hydrolysis of **2** with NaOH 0.1 M. Then, 4.29 g (1.16×10^{-3} mol) of **2** is dissolved in 240 mL of NaOH 1 M and stirred at 55 °C during 24 h. Then the solvent is evaporated, and the polymer is dissolved in dichloromethane, dried with $MgSO_4$, filtered, concentrated, precipitated in diethyl ether, filtered, and dried under vacuum.

81% yield; 75% functionalization.

^{1}H NMR data in $CDCl_3$: δ (ppm) 1.08−1.79 (m, 4H, $-CH_2-_{cycle}$); 2.72−3.45 (m, 3H, $-CH-_{cycle}$); 3.63 (m, 405H, $-CH_2-O-$); 4.08 (s, 1.5H, $-O-CH_2-COOH$); 5.91−6.09 (m, 2H, $-CH=CH-_{cycle}$).

Synthesis of α-Norbornenyl-ω-acetal Poly(ethylene oxide). α-Norbornenyl-ω-acetal poly(ethylene oxide) (**4**) is prepared by nucleophilic substitution of **1** on bromoacetaldehyde diethyl acetal activated by NaH. The same protocol as for **2** is followed.

81% yield; 100% functionalization.

^{1}H NMR data in $CDCl_3$: δ (ppm) 1.08−1.79 (m, 4H, $-CH_2-_{cycle}$); 1.19 (t, J = 7 Hz, 6H, $-CH_3$) 2.72−3.45 (m, 3H, $-CH-_{cycle}$); 3.63 (m, 340H, $-CH_2-O-$);4.61 (t, J = 5 Hz, 1H $-CH-$); 5.91−6.09 (m, 2H, $-CH=CH-_{cycle}$).

Synthesis of α-Norbornenyl-ω-GS Poly(ethylene oxide). α-Norbornenyl-ω-GS poly(ethylene oxide) (**5**) is obtained in two steps. First, **4** is hydrolyzed to give α-norbornenyl-ω-aldehyde poly(ethylene oxide). Then, 4.05 g of (4) (1.1×10^{-3} mol) is dissolved in 50 mL of HCl 6N and stirred during 6 h at room temperature. In a second step, aldehyde reacts with an amine group of GS to produce **5**. For that, 400 mL of buffer solution pH 12 and NaOH pellets are added to the solution to adjust the pH. Then, 3.15 g of GS (5 eq.; 5.5×10^{-3} mol) dissolved in 15 mL of buffer solution is added dropwise. The mixture is stirred at room temperature during 12 h. Then the solvent is evaporated, and the polymer is dissolved in dichloromethane, dried with $MgSO_4$, filtered, concentrated, precipitated in diethyl ether, filtered, and dried under vacuum. The two steps are carried out in one-pot. The product is purified by a three-day dialysis and lyophilized.

82% yield; 100% functionalization.

^{1}H NMR data in D_2O: δ (ppm) = 1.18−3.28 (m, 35H, GS and Nb protons); 2.83 (s, 3H, CH_3-HN- GS) 3.74 (m, 404H, $-CH_2-O-$); 5.15 (m, 1H, $-CH-_{cycle}$ GS); 5.61 (m, 1H, $-CH-_{cycle}$ GS); 5.91−6.09 (m, 2H, $-CH=CH-_{cycle}$).

Macromonomers are stored under argon atmosphere in a glovebox.

NPs Synthesis. Functionalized NPs are formed by ROMP in dispersion. Dispersion polymerizations are carried out at room temperature under inert atmosphere (glovebox) and stirring. Solvents are degassed according to the freeze−pump−thaw procedure. In a typical synthesis, 30 mg (3.6×10^{-5} mol) of Grubbs first generation complex are dissolved in 5 mL of dichloromethane/ethanol mixture (50/50% vs volume). Both norbornene (6.1×10^{-3} mol) and macromonomer (1.5×10^{-4} mol) are first dissolved in 18 mL of dichlomethane/ethanol solution (35/65% v/v) and added to the catalyst. The mixture is stirred during 24 h. At the end of polymerization Ruthenium end-capped chains are deactivated by addition of 0.3 mL of ethyl vinyl ether.

Then the NPs are transferred in aqueous medium for bacterial tests: first, dichloromethane was evaporated, then water was added dropwise and ethanol was evaporated. Finally, the NPs dispersion was purified by ultrafiltration with five times the initial volume. After this treatment no residual ruthenium complex is observed, as proved by elemental analysis.

Cytotoxicity. The noncytotoxicity of NPs has been shown by Neutral Red and MTT essays.[31]

MIC Measurements. Minimum inhibitory concentrations (MIC) of a freshly prepared GS solution is compared to GS released solutions obtained from GS-functionalized macromonomer or GS-functionalized NPs after 6 or 16 h in buffer solutions at pH 4, 5, 6, or 7. A bacterial culture is prepared by mixing a Mueller−Hinton (MH) broth (Conda S.A.) with a *S. epidermidis* strain (ATCC number 12228), allowing the bacterial proliferation overnight in an incubator at 37 °C. The bacterial culture is then diluted with MH broth in order to obtain a turbidity at 600 nm of OD_{600} = 0.6 corresponding to 10^8 cfu·mL^{-1}. From a 640 μg/mL solution of GS, a serial dilution is performed to which 100 μL of bacterial culture diluted 100 times with MH broth are added resulting in a final GS concentration range of 256−0.0625 μg·mL^{-1} and a bacterial suspension of 10^5 cfu. We checked that pH solution was around 7 after dilutions and before introduction of bacteria even if we used at first acidic buffer solutions. A similar dilution series is prepared using buffer solutions in which GS has been released from macromonomer and NPs (initial concentrations have been determined in GS equivalent: concentration of GS free or linked). The MIC was considered the lowest concentration of the sample that prevents visible growth after 12 h at 37 °C.

3. RESULTS AND DISCUSSION

Macromonomer Synthesis. α,ω-Functionalized macromonomer synthesis (Scheme 1) is the first stage of the NPs formation. It requires five steps after each macromonomer has been characterized by ^{1}H NMR spectroscopy, size exclusion chromatography (SEC), MALDI−TOF mass spectrometry, and thin layer chromatography (TLC) in order to check its structures and its functionalization yields.

Synthesis of ***1***. α-Norbornenyl macromonomer **1** was produced by anionic ring-opening polymerization of ethylene oxide initiated by 5-norbornene-2-methanol (NbOH). NbOH was first deprotonated by DPMK to create the active species. Thanks to the thermodynamic equilibrium between the active species R-O$^-$ and the inactive species R−OH, the anionic polymerization of EO is perfectly controlled and involve to a macromonomer with a narrow distribution characterized by a polydispersity lower than 1.1 (Table 1 entry 1). ^{1}H NMR has been carried out in order to check its structure (Figure 2 (1)) and to calculate the polymerization degree ($\overline{DP}_n$) using the integration of the ethylenic protons of the norbornenyl group (I_{Nb}) (δ = 5.91, 6.04, and 6.09 ppm (2H)) and the protons of the PEO linear chain (I_{PEO}) (δ = 3.63 ppm) ($4\overline{DP}_nH$). By this way, the number-average molecular weights ($\overline{M}_n$) has also been calculated

Scheme 1. Synthesis of *α,ω*-Functionalized Macromonomers

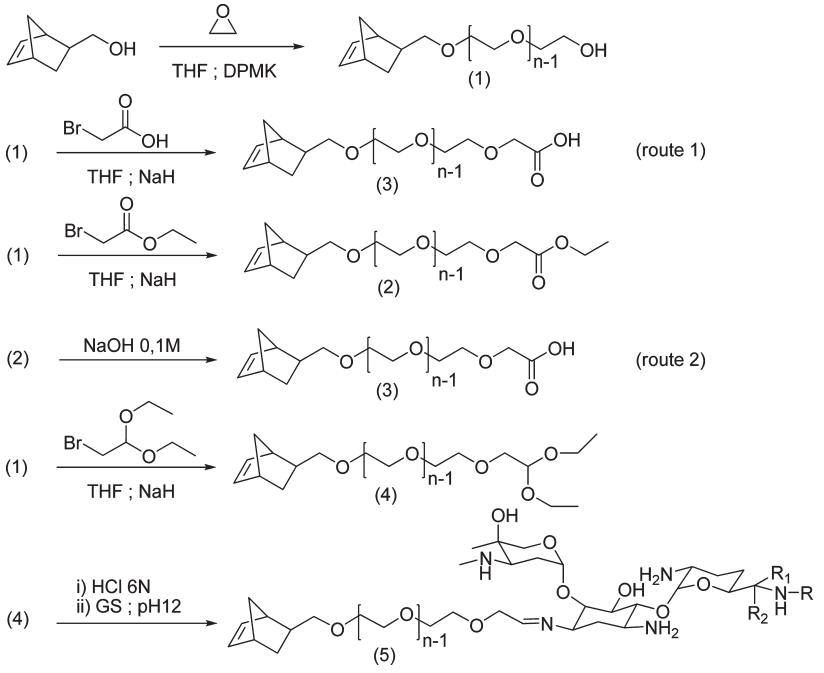


Table 1. Characteristics of *α,ω*-Functionalized Macromonomers

entry	macrom	mass yields (%)	f (%)[c]	$\overline{M}_{n,calculated}$[b] (^{1}H NMR) (g mol^{-1})	$\overline{DP}_{n,calculated}$[a] (^{1}H NMR)	$\overline{M}_{n,experimental}$ (SEC) (g mol^{-1})	PDI (SEC)	MALDI TOF (m/z) (g mol^{-1})
1	**1**	83	-	3640	83	3750	1.09	3516.1 ± 44.0n
2	**2**	86	85	4050	92	4560	1.12	3692.2 ± 44.0n
								3822.2 ± 44.0n
3	**3**[d]	87	15	4250	96	4360	1.15	3956.3 ± 44.0n
								3947.3 ± 44.0n
4	**3**[e]	81	75	4120	93	4440	1.20	3648.2 ± 44.0n
								3727.2 ± 44.0n
5	**4**	81	>99	4130	93	3720	1.08	3984.3 ± 44.0n
6	**5**	82	>99	4640	92	4270	1.18	4082.5 ± 44.0n
								4090.5 ± 44.0n
								4097.4 ± 44.0n

[a] $\overline{DP}_n = I_{PEO}/2I_{Nb}$ with I_{PEO} = integration of the protons of the PEO linear chain, and I_{Nb} = integration of the ethylenic protons of the norbornenyl group. [b] $\overline{M}_n = 44\overline{DP}_n + M_{Nb} + M_\omega$ with M_{Nb} = molecular weight of the norbornenyl group and M_ω = the molecular weight of the ω-function; 44 is the molecular weight of the ethylene oxide unit. [c] f: functionalization yields $f(\mathbf{2}) = I_{ester}/2I_{Nb}$; $f(\mathbf{3}) = I_{acid}/I_{Nb}$; $f(\mathbf{4}) = 2I_{Ac}/I_{Nb}$; $f(\mathbf{5}) = 2I_{GS}/I_{Nb}$. [d] Obtained from route 1. [e] Obtained from route 2.

($\overline{M}_n$ = 3750 g·mol^{-1}) and was confirmed by the SEC measurement ($\overline{M}_n$ = 3640 g·mol^{-1}) and the MALDI TOF analysis (m/z = 3516.1 ± 44.0n g mol^{-1}) (Table 1, entry 1). This correlation between the results of the three analyses permitted to prove the presence of norbornenyl entities on each macromolecule and consequently the ability for each chain to react in ROMP.

*Synthesis of (**3**).* The carboxylic acid functionalization was carried out in two ways. In a first strategy (Scheme 1, route 1), *α*-norbornenyl-*ω*-carboxylic acid poly(ethylene oxide) (3) was synthesized by nucleophilic substitution of (1) - deprotonated by sodium hydride - on the bromo-acetic acid. The product was characterized by ^{1}H NMR, allowing first to ensure the nondegradation of the norbornenyl entities (the ratio between the integration of the ethylenic protons of the norbornenyl group and the integration of the proton of the PEO linear chain remained constant), and second to determine the *ω*-functionalization yield thanks to the ratio between the integration of the protons of the *ω* group (I_{acid}) (δ = 4.08 ppm ; 2H) and the ethylenic protons of the norbornenyl group (I_{Nb}) using the eq a.

$$f(3) = \frac{I_{acid}}{I_{Nb}} \qquad \text{(a)}$$

The low increases of the molecular weight between **1** and **3** are explained by the successive precipitations and the conservation of only the higher masses. With this first strategy, a functionalization degree of only 15% was obtained (Table 1 entry 3). This low

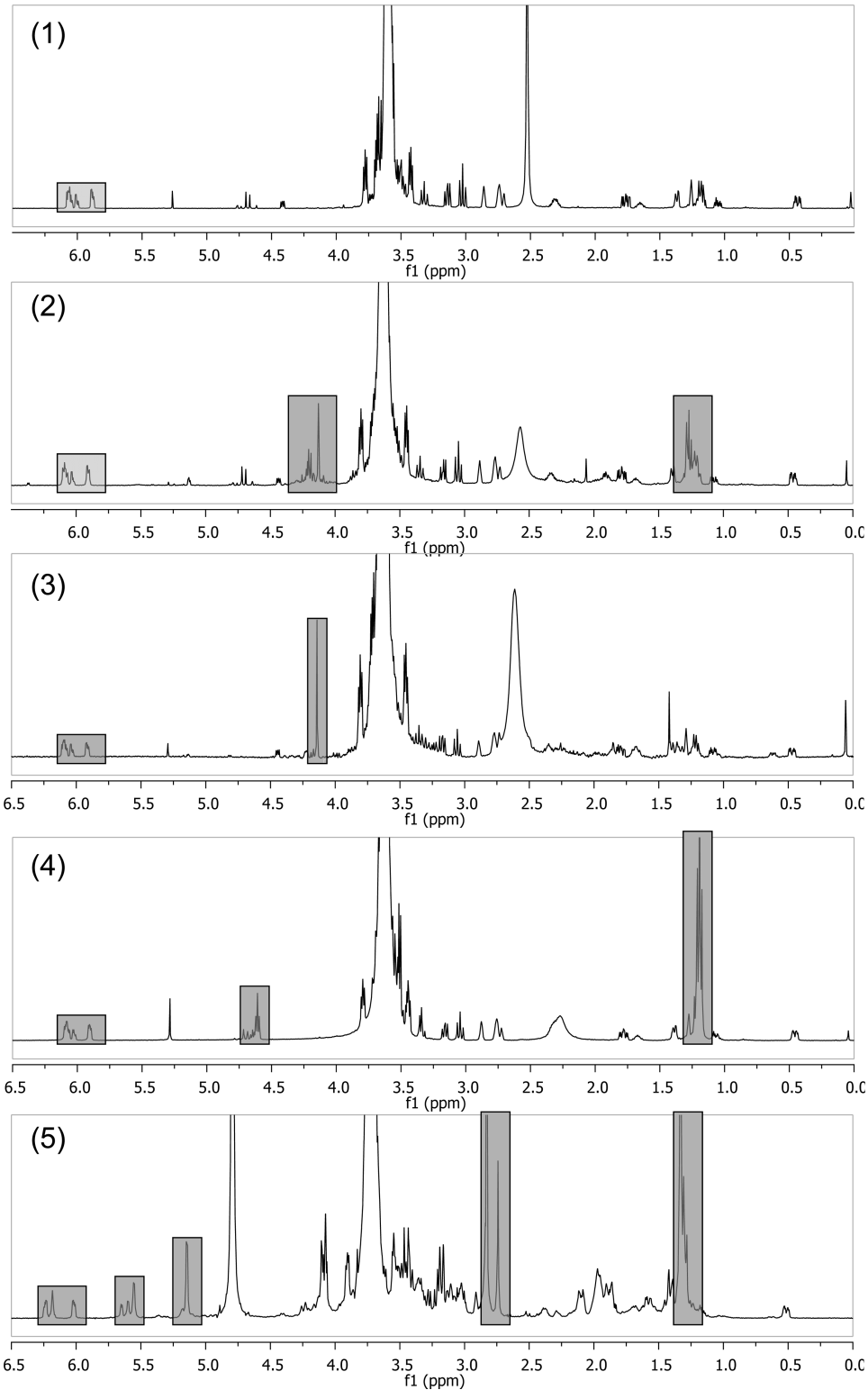
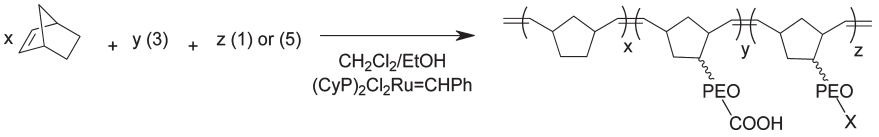


Figure 2. ^{1}H NMR spectra for the five macromonomers.

value can be explained by lower reactivity of brominated derivative due to the presence of a carboxylic acid group. Indeed, the excess of sodium hydride (pK_a = 35) using to deprotonate the alcohol (pK_a = 16−17) reacts with the carboxylic acid group to form a carboxylate (pK_a = 4−5). The carboxylate derivative exhibits a lower electrophilicity and subsequently a lower reactivity by nucleophilic substitution. That is why a second strategy (Scheme 1 route 2) has been preferred, consisting to a nucleophilic substitution on a bromo-ester, then a hydrolysis of the ester to give acid derivative.[32]

α-Norbornenyl-ω-ester poly(ethylene oxide) (**2**) was synthesized by nucleophilic substitution of **1** deprotonated by sodium

Scheme 2. Synthesis of Polynorbornene−Poly(ethylene oxide) NPs, −X = −OH or −GS



hydride on the 2-bromoethyl acetate. As for **3**, ^{1}H NMR analysis of **2** (Figure 2(2)) has permitted us to calculate its number-average molecular weight, approximately the same that the number-average molecular weight measured by SEC, confirming the total presence of the norbornenyl group (Table 1 entry 2). It also allows determining the functionalization yield using the integration of the $-CH_2-$ protons of the ester group (I_{ester}) (δ = 4.13 (2H) and 4.20 ppm (2H)) and the integration of the ethylenic protons of the norbornenyl groups (I_{Nb}) with the eq b.

$$f(2) = \frac{I_{ester}}{2I_{Nb}} \quad \text{(b)}$$

2 was obtained with a functionalization yield of 85%. MALDI TOF analysis presented two populations corresponding to **1** and **2**.

2 has consequently been hydrolyzed with soda solution 0.1 M to obtain **3**. The comparison between ^{1}H NMR (Figure 2(3)) and SEC (Table 1 entry 4) analysis of **3** also proved the preservation of the norbornenyl entities and allowed determining the functionalization yield, which was 75%, much better than for direct functionalization with the bromo acid. MALDI−TOF mass spectrometry and TLC analysis have also been carried out on this macromonomer. On the MALDI−TOF spectrogram, two populations are present corresponding to nonfunctionalized macromonomer **1** and ω-carboxylic acid macromonomer **3** (Table 1 entry 4). No trace of **2** has been observed attesting to the hydrolysis efficiency. Similar results were observed in TLC.

Synthesis of ***5***. The synthesis of α-norbornenyl-ω-acetal poly(ethylene oxide) (**4**) is the first step of the GS functionalization. **4** is obtained by nucleophilic substitution of **1** deprotonated by sodium hydride on bromoacetaldehyde diethyl acetal. ^{1}H NMR analysis allowed confirming the formation of **4** with the appearance of new peaks (δ = 1.19 (6H) and 4.61 ppm (1H)) characteristic of an acetal group (Figure 2 (4)). The relatively good agreement between experimental and calculated molar masses associated with a functionalization yield close to unity (Table 1 entry 5) proves the efficiency and the selectivity of the reaction. This result is confirmed by TLC and by MALDI−TOF with the presence of only one population corresponding to **4**.

Finally, GS-functionalized macromonomer **5** is synthesized in two steps thanks to **4**. First **4** is activated into aldehyde by acidifying the medium (pH < 1) with HCl, then the grafting of drug onto the macromonomer is carried out. The aldehyde group reacts with an amine function GS to link the drug on the macromonomer through a pH-sensitive imine bond. The GS is a molecule bearing three primary amines and two secondary amines. The pH is adjusted (pH = 12) before the addiction of GS in order to have the deprotonated form of the molecule. Because of the low stability of aldehyde derivatives, the two steps have been carried out in one-pot. ^{1}H NMR (Figure 2(5)) analysis of **5** is in consistence with the desired structure: we observed the presence of the norbornenyl groups and characteristic peaks[33] of GS (δ = 5.15 ppm, 1H; δ = 5.61 ppm, 1H protons of the GS cycles and 2.6 < δ < 2.8 protons of methyl groups linked on amine functions of GS). The high functionalization yield (f(**5**) > 99%), determined using I_{Nb} and the integration of a characteristic peak of GS (I_{GS}) (δ = 5.61 ppm, 1H) with the eq d, demonstrates that GS has been linked on the macromonomer quantitatively.

$$f(\mathbf{5}) = \frac{2I_{GS}}{I_{Nb}} \quad \text{(d)}$$

In addition, MALDI−TOF mass spectrometry analysis of the GS-macromonomer (Table 1 entry 6) shows three populations corresponding to the three forms of GS (C1, C1a, and C2)[33] linked on the macromonomer with an imine bond. **5** has equally been characterized by TLC, and the presence of only one spot proves that GS is grafted on the macromonomer and there is no free GS anymore.

NPs Synthesis. NPs were obtained by ROMP in dispersion in a mixture of dichloromethane/ethanol (35/65 V/V). The reaction was initiated by the first generation Gubbs complex. Nb and PEO macromonomers are both soluble in the solvent. The polynorbornene, not soluble in the medium, precipitates to form NPs with hydrophobic core and hydrophilic PEO shell. This reaction is described in Scheme 2.

In this part, we have drawn particular attention to the influence of functional groups bearing by the macromonomers on the NPs stability and size as a function of the solvent. Indeed, NPs are synthesized in a mixture of dichloromethane/ethanol, have to be transferred in aqueous medium for the *in vitro* evaluation tests. Four batches of NPs have been synthesized using only one macromonomer or mixtures of them in equimolar proportion. For each batch, Nb and macromonomer conversions have been determined respectively by GC and SEC measurement and the NPs sizes were determined by DLS measurements in different solvents (Table 2).

In each case, a quasi-total conversion was observed (π > 95% for Nb and π > 90% for macromonomers). These results prove the high reactivity of such systems. Thanks to these high conversions, we were able to evaluate the concentrations of GS and carboxylic acid in the dispersion: For NP_2, the GS concentration was about 1.4×10^{-2} mol L^{-1} and, for NP_4, the GS and carboxylic acid concentrations were about 7×10^{-3} mol L^{-1}. Because of its hydrophilic properties, GS is essentially located on the particle shells.

Colloidal stability of NPs has been shown by measuring the NPs diameters. The diameter values measured by DLS were the z-average diameters, that is to say the mean diameters based on the intensity of scattered light. Measures have been carried out in the reaction solvent (dichloromethane/ethanol 35/65), then NP_1 and NP_2 were transferred in aqueous medium in order to use it for the *in vitro* bacterial inhibition tests. In our experimental conditions, NPs had *z*-average diameters of about 350 nm in dichloromethane/ethanol with a narrow distributions (polydispersity index lower than 0.3) whatever the functionalizations. The different functionalizations do not seem to have significant influence on the *z*-average diameters of the NPs (from 300 to 360 nm). For NP_1 and NP_2, diameters increased in water.

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Table 2. Conversion of Nb and Macromonomers for the ROMP Reactions and *z*-Average Diameters of the NPs

				z-average diameters of NPs (nm) (DLS) and polydispersity index	
	macromonomers[a]	macromonomer conversion (%)[c]	Nb conversion (%)[d]	in $EtOH/CH_2Cl_2$	in H_2O
NP_1	**1**	>99	98	320 (0.20)	410 (0.27)
NP_2	**5**	92	>99	360 (0.21)	395 (0.25)
NP_3	**1** and **3**[b]	96	96	300 (0.18)	-
NP_4	**5** and **3**[b]	94	>99	345 (0.17)	-

[a] [Macromonomers]/[Nb] = 0.025. [b] In equimolar proportions. [c] Measured by SEC with dodecane as internal standard. [d] Measured by GC with dodecane as internal standard.

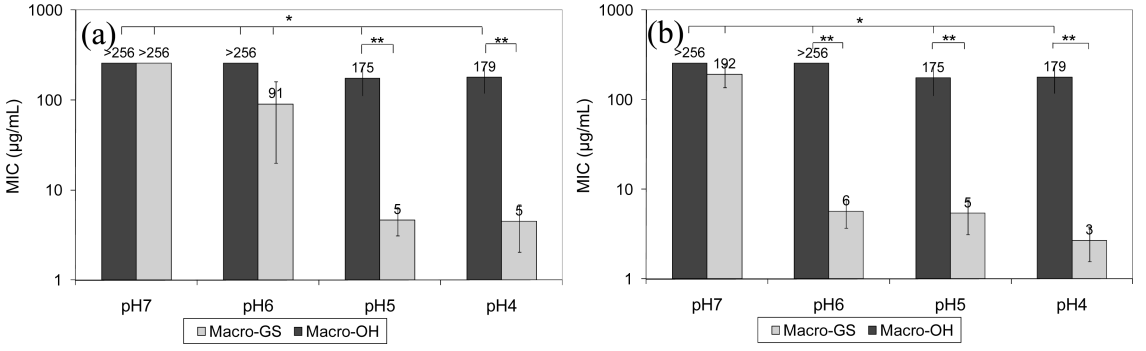

Figure 3. MIC measurements of GS-functionalized macromonomer after 6 h (a) and 16 h (b) of GS release in buffer solutions. Nonfunctionalized macromonomer is used as control. Bacterial strain was *S. epidermidis* 10^5 cfu. In the same conditions, MIC for GS < 0.5 $\mu g \cdot mL^{-1}$.

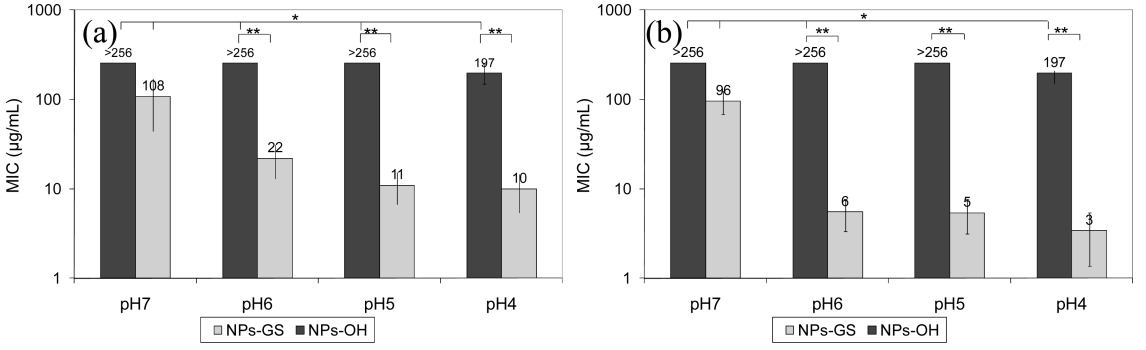

Figure 4. MIC measurements of GS-functionalized NPs after 6 h (a) and 16 h (b) of GS release in buffer solutions. Nonfunctionalized NPs are used as control. Bacterial strain was *S. epidermidis* 10^5 cfu. In the same conditions, MIC for GS < 0.5 $\mu g \cdot mL^{-1}$.

This phenomenon can be interpreted by an increase of the solvent polarity, involving a swelling of the poly(ethylene oxide) shell. The presence of GS highly soluble in water does not seem to affect the NPs diameter. Anyway, these measurements proved first the ability to synthesize functionalized NPs (with carboxylic acid groups or GS), and second, we demonstrated their stabilities in aqueous medium for the *in vitro* activity.

***In vitro* Activity Evaluation.** GS, as numerous aminoglycoside antibiotics, is effective against a wide range of Gram negative and some Gram positive organisms including *staphylococci*. It acts on the protein synthesis by binding to the bacterial ribosomes.[20,34] The antibacterial activity evaluation of the system has been carried out through MIC measurements using *S. epidermidis* (10^5 cfu) as bacterial strain. In these experimental conditions, MIC of GS was less than 0.5 $\mu g \cdot mL^{-1}$. The interest of these tests was first to evaluate the GS release at acidic pH and second to prove the *in vitro* antibacterial activity of such a system. The pH decrease in case of infection, due to massive infiltration of neutrophils and macrophages to the site of infection[25] can only be observed in physiological medium. That is why the acidic pH has been artificially reproduced by using buffer pH solution for the *in vitro* experiments. The activities of GS-functionalized macromonomer (**5**) and GS-functionalized NPs (NP_2) have been assessed. In each case, nonfunctionalized products have been used as control (**1** and NP_1) Macromonomers and NPs have been introduced in buffer pH solutions (pH = 4, 5, 6 and 7) and kept at room temperature for 6 or 16 h in order to release GS, and then MIC measurements have been carried out. In order to eliminate the initial turbidity, NPs solutions have been filtered at 0.1 μm before measurements. We have paid particular attention to the influence for buffer pH solutions and more particularly the presence of salts from them on the bacterial growth. A wide range of

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concentration of salts has been tested and no influence has been observed (results not shown). Figure 3 presents the MIC of macromonomer solutions as a function of pH for 6 and 16 h of release.

Each test has been carried out three times and the standard deviations are presented on the graphs. In our experimental conditions, the higher concentration in GS is 256 $\mu g \cdot mL^{-1}$, that is why, when no inhibition was observed, this value was attributed to the test. For the nonfunctionalized macromonomer, used as control, no significant inhibition is observed whatever the pH. For GS-functionalized macromonomer no significant inhibition was recorded at pH = 7 (Mann and Whitney tests $p > 0.05$), while a decrease of MIC appeared at pH = 5 and 4. At pH = 6, a longer time (16 h, Figure 3b) is necessary to observe the imine bond cleavage and a significant decrease of the MIC ($p < 0.025$). Regarding the results of NPs series (Figure 4), it shows a similar profile with no inhibition of the control NPs and a decrease of the MIC as a function of pH for GS-functionalized NPs. No significant activity was observed at pH = 7 ($p > 0.05$). With these experiments, we wanted to prove the activity of our system in the beginning of the release (6 and 16 h). Indeed, for these durations, less than 10% of the GS has been released, explaining the higher values of MIC comparing to free GS (MIC > 3 $\mu g \cdot mL^{-1}$ vs <0.5 $\mu g \cdot mL^{-1}$ for free GS). Higher release percentage of GS (>80%) are observed after 5 to 7 days (results not shown).

The pH-sensitive GS-functionalized NPs we present in this paper are designed to be used as local antibacterial drug delivery systems after their grafting onto biomaterials. The pH control of drug release, commonly used for the tumor treatments, has never been employed for this kind of application. Indeed, the drug delivery systems of antibiotics typically present in the literature are either noncontrolled, and release drugs continuously,[33,35] or thermally controlled.[36,37] For the latter systems, drugs are commonly contained in hydrogel networks and released in case of an increase of temperature.[38] The downsides of such a process are first, that a part of the drug is trapped inside the polymer networks and is never released and second, these systems offer an on−off release regulation. With our system, all the active molecules located on the surfaces of the NPs, are potentially able to be released. Second, with the pH control and more particularly the imine pH-sensitive bond, drugs are released only if necessary and the drug release kinetics are modulated by the pH decrease. The more acute the infection and consequently the lower the pH are, the faster the drug is released.

4. CONCLUSION

In this work, we have presented the synthesis of NPs functionalized with an antibiotic, Gentamicin Sulfate. These objects are designed to allow the release of drug at acidic pH though a pH-sensitive link—typically in case of infection—and to permit their anchoring onto biomaterial surfaces. Macromonomers and NPs syntheses and their chemical characterizations were described and we have proved the ability to use them as a pH-controlled drug delivery system. In a second part, their *in vitro* antibacterial activities after release of the antibiotic at acidic pH have been demonstrated by MIC measurements. We were able to observe a significant inhibition of bacterial growth after release of GS from pH = 6.

In vitro activity of GS-functionalized NPs after release of the antibiotic only at acidic pH has been validated. Consequently, NPs grafting on biomaterials and their antibacterial activities are currently in progress. *In vivo* experiments are equally in progress on rabbits.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +33 (0) 5 40 00 65 74. Fax: +33 (0) 5 40 00 84 87. E-mail: heroguez@enscbp.fr.

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REFERENCES

(1) Zhang, L.; Gu, F. X.; Chan, J. M.; Wang, A. Z.; Langer, R. S.; Farokhzad, O. C., *Nanoparticles in Medicine: Therapeutic Applications and Developments*; Nature Publishing Group: Basingstoke, U.K., 2008; Vol. 83, p 9.

(2) Martin del Valle, E. M.; Galan, M. A.; Carbonell, R. G. *Ind. Eng. Chem. Res.* **2009**, *48* (5), 2475–2486.

(3) Gao, W.; Chan, J. M.; Farokhzad, O. C. *Mol. Pharmaceutics* **2010**, *7* (6), 1913–1920.

(4) Ulbrich, K.; Subr, V. *Adv. Drug Delivery Rev.* **2004**, *56* (7), 1023–1050.

(5) Asokan, A.; Cho, M. J. *J. Pharm. Sci.* **2002**, *91* (4), 903–913.

(6) Gerweck, L. E.; Vijayappa, S.; Kozin, S. *Mol. Cancer Ther.* **2006**, *5* (5), 1275–1279.

(7) Sant, V. P.; Smith, D.; Leroux, J.-C. *J. Controlled Release* **2004**, *97* (2), 301–312.

(8) Lee, E. S.; Oh, K. T.; Kim, D.; Youn, Y. S.; Bae, Y. H. *J. Controlled Release* **2007**, *123* (1), 19–26.

(9) Xue, Y.-N.; Huang, Z.-Z.; Zhang, J.-T.; Liu, M.; Zhang, M.; Huang, S.-W.; Zhuo, R.-X. *Polymer* **2009**, *50* (15), 3706–3713.

(10) Liu, Y.; Cao, X.; Luo, M.; Le, Z.; Xu, W. *J. Colloid Interface Sci.* **2009**, *329* (2), 244–252.

(11) Ma, L.; Liu, M.; Liu, H.; Chen, J.; Cui, D. *Int. J. Pharm.* **2010**, *385* (1−2), 86–91.

(12) Arias, J. L.; Linares-Molinero, F.; Gallardo, V.; Delgado, Á. V. *Eur. J. Pharm. Sci.* **2008**, *33* (3), 252–261.

(13) Chan, Y.; Bulmus, V.; Zareie, M. H.; Byrne, F. L.; Barner, L.; Kavallaris, M. *J. Controlled Release* **2006**, *115* (2), 197–207.

(14) Chen, W.; Meng, F.; Cheng, R.; Zhong, Z. *J. Controlled Release* **2010**, *142* (1), 40–46.

(15) Jung, J.; Lee, I.-H.; Lee, E.; Park, J.; Jon, S. *Biomacromolecules* **2007**, *8* (11), 3401–3407.

(16) Gu, J.; Cheng, W.-P.; Liu, J.; Lo, S.-Y.; Smith, D.; Qu, X.; Yang, Z. *Biomacromolecules* **2007**, *9* (1), 255–262.

(17) Ding, C.; Gu, J.; Qu, X.; Yang, Z. *Bioconjugate Chem.* **2009**, *20* (6), 1163–1170.

(18) Shen, Y.; Jin, E.; Zhang, B.; Murphy, C. J.; Sui, M.; Zhao, J.; Wang, J.; Tang, J.; Fan, M.; Van Kirk, E.; Murdoch, W. J. *J. Am. Chem. Soc.* **2010**, *132* (12), 4259–4265.

(19) Aryal, S.; Hu, C.-M. J.; Zhang, L. *ACS Nano* **2009**, *4* (1), 251–258.

(20) Chang, H, I.; Perrie, Y.; Coombes, A. G, A. *J. Controlled Release* **2006**, *110* (2), 414–421.

(21) Ouchi, T.; Kuroda, H.; Ohya, Y. *ACS Symp. Ser.* **1997**, *680* (19), 284–296.

(22) Md, B. B. *Nutr. Rev.* **2007**, *65*, S170–S172.

(23) Marnell, L.; Mold, C.; Du Clos, T. W. *Clin. Immunol.* **2005**, *117* (2), 104–111.

(24) Arenberg, D. A., Strieter, R. M. In *Angiogenesis. Inflammation: Basic Principles and Clinical Correlates*. Gallin, J. I., Snyderman, R., Eds.; Lippincott, Williams, and Wilkins: Philadelphia, PA, 1999.

(25) Issekutz, A. C.; Bhimji, S. *Infect. Immunol.* **1982**, *36* (2), 558–566.

(26) Heroguez, V.; Breunig, S.; Gnanou, Y.; Fontanille, M. *Macromolecules* **1996**, *29* (13), 4459–4464.
(27) Quemener, D.; Chemtob, A.; Heroguez, V.; Gnanou, Y. *Polymer* **2005**, *46*, 1067–1075.
(28) Schoifet, S. D.; Morrey, B. F. *J. Bone Joint Surg.* **1990**, *72* (9), 1383–1390.
(29) Bertin, P. A.; Gibbs, J. M.; Shen, C. K.-F.; Thaxton, C. S.; Russin, W. A.; Mirkin, C. A.; Nguyen, S. T. *J. Am. Chem. Soc.* **2006**, *128* (13), 4168–4169.
(30) Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E. *Angew. Chem., Int. Ed.* **2006**, *45* (15), 2348–2368.
(31) Durrieu, M. C.; Quemener, D.; Baquey, C.; Sabaut-Heroguez, V. Patent WO2006008386, 2006.
(32) Bückmann, A. F.; Morr, M.; Johansson, G. *Makromol. Chem.* **1981**, *182* (5), 1379–1384.
(33) Lesniak, W.; McLaren, J.; Harris, W. R.; Pecoraro, V. L.; Schacht, J. *Carbohydr. Res.* **2003**, *338* (24), 2853–2862.
(34) Block, S. S. *Disinfection, sterilization, and preservation*. Lippincott Williams & Wilkins: Philadelphia, PA, 2001; p 1481.
(35) Habraken, W. J. E. M.; Wolke, J. G. C.; Jansen, J. A. *Adv. Drug Delivery Rev.* **2007**, *59* (4−5), 234–248.
(36) Anal, A. K. *Recent Pat. Endocr., Metab. Immune Drug Discovery* **2007**, *1*, 83–90.
(37) Al-Abd, A. M.; Hong, K. Y.; Song, S. C.; Kuh, H. J. *J. Controlled Release* **2010**, *142* (1), 101–107.
(38) Peng, K. T.; Chen, C. F.; Chu, I. M.; Li, Y. M.; Hsu, W. H.; Hsu, R. W. W.; Chang, P. J. *Biomaterials* **2010**, *31* (19), 5227–5236.