Synthesis of Highly Functionalized Poly(alkyl cyanoacrylate) Nanoparticles by Means of Click Chemistry

Julien Nicolas,*,† Fethi Bensaid,† Didier Desmaële,‡ Mathurin Grogna,§ Christophe Detrembleur,§ Karine Andrieux,† and Patrick Couvreur†

Laboratoire de Physico-Chimie, Pharmacotechnie et Biopharmacie, Univ. Paris-Sud, UMR CNRS 8612, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry, France, Laboratoire Biocis, Univ. Paris-Sud, UMR CNRS 8076, Faculté de Pharmacie, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry, France, and Center for Education and Research on Macromolecules (CERM), University of Liège, Sart-Tilman, B6, 4000 Liège, Belgium

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ABSTRACT: A general methodology was proposed to prepare highly functionalized poly(alkyl cyanoacrylate) nanoparticles by means of Huisgen 1,3-dipolar cyclo-addition, the so-called click chemistry. To achieve this goal, different protocols were investigated to obtain azidopoly(ethylene glycol) cyanoacetate of variable molar mass, followed by a Knoevenagel condensation−Michael addition reaction with hexadecyl cyanoacetate to produce a poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-N_3PEGCA)) copolymer, displaying azide functionalities at the extremity of the PEG chains. As a proof of concept, model alkynes were quantitatively coupled either to the P(HDCA-*co*-N_3PEGCA) copolymers in homogeneous medium followed by self-assembly in aqueous solution or directly at the surface of the preformed P(HDCA-*co*-N_3PEGCA) nanoparticles in aqueous dispersed medium, both yielding highly functionalized nanoparticles. This versatile approach, using alkyl cyanoacrylate derivatives, opened the door to ligand-functionalized and biodegradable nanoparticles with "stealth" properties for biomedical applications.

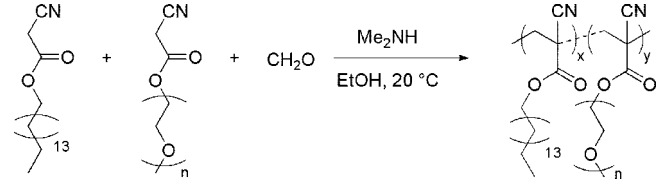
Introduction

Nanoparticles developed from poly(alkyl cyanoacrylate) (PACA) biodegradable polymers have opened new and exciting perspectives in the field of drug delivery due to their nearly ideal characteristics as drug carriers in connection with biomedical applications. Introduced more than 25 years ago in the field of pharmacology,[1] PACA drug carriers have, indeed, demonstrated significant advantages for the treatment of numerous pathologies such as cancer[2] and severe infections (viral, bacteriologic, parasite)[3] as well as several metabolic and autoimmune diseases,[4] which has been well-reviewed in the recent literature.[5−7] Clinical trials have even shown that these nanodevices are safe and biocompatible when loaded with the anticancer drug doxorubicin.[8]

Throughout the last two decades, PACA nanoparticles with different features have been developed:[9] nanospheres (matrix-type nanoparticles),[1,10−15] nanocapsules (vesicular-type nanoparticles) either oil- or water-containing,[16−25] as well as nanoparticles with controlled-surface properties;[14,22,26−39] the later being considered as the second generation of drug delivery devices. Regarding this recent class of advanced PACA nanoparticles, the major breakthrough is undoubtedly the grafting of poly(ethylene glycol) (PEG), termed "PEGylation". PEG is a hydrophilic and flexible polymer intensively employed in the pharmaceutical area, especially for drug delivery purposes such as polymer-protein/peptide bioconjugates[40−45] or long-circulating nanoparticles.[28,46−48] Indeed, PEG gives rise to several potential beneficial effects including increased bioavailability and plasma half-lives, biocompatibility/decreased immunogenicity, reduced proteolysis and enhanced solubility and stability, thus being considered as a key material in this field.[40] Considering nanoparticle technology, non-"PEGylated" nanoparticles are quickly eliminated from the bloodstream due to the adsorption of blood proteins (opsonins) onto their surface, which triggers the recognition by the macrophages of the mononuclear phagocyte system (MPS). As a consequence, these nanoparticles accumulate in the organs of the MPS such as the liver and the spleen, restricting the therapeutic activity of the entrapped compounds to hepatic diseases. In contrast, when covered by PEG chains, the obtained nanoparticles are able to efficiently escape this recognition system, resulting in long-circulating colloidal devices, also called "stealth" nanoparticles.[46,48]

Alkyl cyanoacrylates monomers are also well-known for their very high reactivity and the excellent adhesive properties of the resulting polymers. However, this unique feature tends to make the synthesis of well-defined and/or functionalizable poly(alkyl cyanoacrylate) architectures extremely difficult or even impossible. A significant step was accomplished to circumvent this important drawback via the synthesis of random poly[(hexadecyl cyanoacrylate)-*co*-methoxypoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-MePEGCA)) comblike copolymers with amphiphilic properties.[28] This original approach derived from tandem Knoevenagel condensation−Michael addition reaction to build the polymeric backbone, where the corresponding cyanoacetates were reacted with formaldehyde in the presence of dimethylamine as the catalyst (Scheme 1).

Scheme 1. Synthesis of Random Poly[(hexadecyl cyanoacrylate)-*co*-methoxypoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-MePEGCA)) Copolymer via Knoevenagel Condensation−Michael Addition Reaction



* Corresponding author: Telephone: +33 1 46 83 58 53. Fax: +33 1 46 61 93 34. E-mail: julien.nicolas@u-psud.fr.

† Laboratoire de Physico-Chimie, Pharmacotechnie et Biopharmacie, Univ. Paris-Sud, UMR CNRS 8612.

‡ Laboratoire Biocis, Univ. Paris-Sud, UMR CNRS 8076.

§ Center for Education and Research on Macromolecules (CERM), University of Liège.

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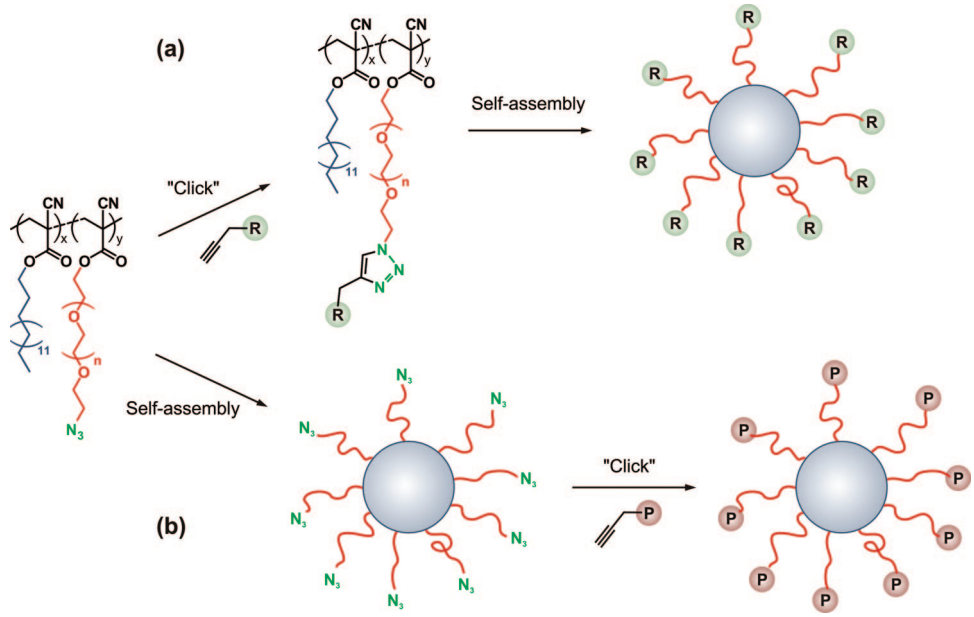


Figure 1. General approach to prepare functionalized poly(alkyl cyanoacrylate) nanoparticles: click reaction on the poly[(hexadecyl cyanoacrylate)-*co*-methoxypoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-N_3PEGCA)) copolymer followed by self-assembly in aqueous solution (a) or click reaction at the surface of preformed P(HDCA-*co*-N_3PEGCA) nanoparticles (b).

However, even though these "PEGylated" nanoparticles have demonstrated a noticeable brain-targeting effect;[6] they suffer from a crucial lack of specificity toward cells and/or tissues and can not be efficiently addressed. Thus, for the forthcoming years, the most exciting challenge in drug delivery, whatever the nature of the drug carriers (i.e., liposome, nanoparticles, etc.), will be undoubtedly the synthesis of efficient ligand-functionalized colloidal devices to achieve specific cells targeting based on a molecular recognition process. To the best of our knowledge concerning PACA technology, only one example of the so-called third generation PACA nanoparticles has been reported and involves poly[(hexadecyl cyanoacrylate)-*co*-aminopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-H_2NPEGCA)) nanospheres displaying folic acid groups,[49,50] to target the folate receptor which is overexpressed at the surface of many tumor cells. However, this approach is restricted to amine-reactive compounds and led to only ~15% folate content at the surface of the nanospheres.[49,50]

In order to extend this concept of functionalizable poly(alkyl cyanoacrylate) "PEGylated" nanoparticles, we have chosen to take advantage of Huisgen 1,3-dipolar cyclo-addition (termed click chemistry)[51,52] between alkyne and azide derivatives due to its high efficiency and its mild experimental conditions.[53−56] Indeed, click chemistry has recently received intense interest as a well-established synthetic route to obtain tailor-made complex materials and has been exploited in many areas such as dendrimers,[57−59] bioconjugates,[60−62] therapeutics[63−65] and functionalized polymers.[66−68]

Herein, we propose a general methodology to obtain highly functionalized PACA biodegradable nanoparticles from a novel poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-N_3PEGCA)) copolymer, as a clickable polymeric scaffold (Figure 1). This versatile approach allowed either: (i) the early coupling reaction to proceed in homogeneous medium with clickable P(HDCA-*co*-N_3PEGCA) copolymers followed by the formation of functionalized nanoparticles by self-assembly in aqueous solution (Figure 1a) or (ii) an effective coupling reaction directly at the surface of clickable P(HDCA-*co*-N_3PEGCA) nanoparticles (Figure 1b). Depending on the characteristics of the desired alkyne moiety (nature, solubility, size, etc.), one of the pathways would be more appropriate than the other one; for instance, if large molecules such as peptide sequences or proteins are required, the click reaction at the surface of the azido-functionalized PACA nanoparticles would be more suitable than early on the copolymer in homogeneous medium, which would undoubtedly alter the following nanoprecipitation process (due to modified hydrophilic−lipophilic balance (HLB)).

In the literature, click chemistry has been employed with nanoparticles based on well-defined poly(acrylic acid)-*b*-polystyrene (PAA-*b*-PS) block copolymers.[69,70] O'Reilly et al. used nitroxide-mediated polymerization (NMP) or reversible addition-fragmentation transfer (RAFT) to prepare shell cross-linked PAA-*b*-PS nanoparticles bearing azide functionalities at their surface, on which an alkyne-fluorescein dye has been successfully clicked.[69] More recently, Opsteen et al. synthesized an azido-terminated PAA-*b*-PS copolymer by atom-transfer radical polymerization (ATRP) to form water-containing nanocapsules covered by azide groups, followed by click reaction with a wide variety of alkyne-ligands based on dansyl dye, biotin, or enhanced green fluorescent protein (EGFP).[70] Even though these two studies clearly demonstrated the feasibility of the click reaction at the surface of these model nanoparticles, a similar approach employing well-established biodegradable polymers such as PACA is highly desirable regarding biomedical applications, where biocompatible and/or biodegradable, ligand-functionalized, colloidal drug carriers are in great demand.

Experimental Section

Materials. Poly(ethylene glycol) monobenzyl ether ($BnPEG_{70}$, $M_{n,NMR}$ = 3160 g·mol^{-1}, $DP_{n,NMR}$ = 70, $M_{n,SEC}$ = 2700 g·mol^{-1}, M_w/M_n = 1.06) was purchased from Polymer Source and used as received. Ethylene oxide (EO, >99%) was purchased from Chemogas. Poly(ethylene glycol) monomethyl ether (PEG_{43}, $M_{n,NMR}$ = 1910 g·mol^{-1}, $DP_{n,NMR}$ = 43, $M_{n,SEC}$ = 1970 g·mol^{-1}, M_w/M_n = 1.04, Fluka), cyanoacetic acid (99%, Fluka), *N*,*N*′-dicyclohexylcarbodiimide (DCC, >99%, Fluka), methanesulfonyl chloride (MsCl, 99.7%, Aldrich), sodium azide (NaN_3, 99.5%, Aldrich), 4-dimethylaminopyridine (DMAP, 99%, Aldrich), formaldehyde (37% in water, Aldrich), pyrrolidine (99%, Aldrich), anhydrous magnesium sulfate (MgSO4, >99%, Aldrich), sodium ascorbate

(Aldrich), copper sulfate pentahydrated ($CuSO_4.5\ H_2O$, >99%, Aldrich), 4-pentyn-1-ol (95%, Acros), triethyl amine (TEA, Aldrich) and *N*-[2-(dimethylamino)ethyl]-*N*′,*N*′,*N*′-trimethyl-1,2-ethanediamine (PMDETA, 99%, Aldrich) were used as received. Copper bromide (CuBr, 97%, Aldrich) was purified according to the method of Keller and Wycoff.[71] 2-Propanol (99.5%) and *N*,*N*-dimethylformamide (DMF) were purchased from Fluka. All other solvents (tetrahydrofuran, (THF), methanol (MeOH), dichloromethane (DCM), diethyl ether (Et_2O), chloroform ($CHCl_3$), ethanol (EtOH), ethyl acetate (EtOAc) and hexane) were purchased at the highest grade from Carlo Erba. Propynyl−dansyl (alkyne−dansyl) was synthesized from dansylchloride (Acros, 98%) and propargyl amine (Acros, 99%) as described elsewhere.[70]

Analytical Techniques. ^{1}H and ^{13}C NMR spectra were performed in deuterated chloroform ($CDCl_3$) or deuterated water (D_2O) at ambient temperature on a Bruker Avance (300 MHz unless otherwise specified and 75 MHz, respectively). IR spectra were obtained on a Fourier Transform Bruker Vector 22 spectrometer. Size exclusion chromatography (SEC) was performed at 30 °C with two columns from Polymer Laboratories (PL-gel MIXED-D; 300 × 7.5 mm; bead diameter: 5 μm; linear part: 400 to 4 × 10^5 g·mol^{-1}) and a differential refractive index detector (Spectrasystem RI-150 from Thermo Electron Corp.). The eluent was $CHCl_3$ at a flow rate of 1 mL·min^{-1} and toluene was used as a flow-rate marker. The calibration curve was based on poly(ethylene glycol) standards (peak molar masses, M_p = 200 to 23 600 g·mol^{-1}) or poly(methyl methacrylate) (PMMA) standards (peak molar masses, M_p = 625 to 625 500 g·mol^{-1}) from Polymer Laboratories. Unless otherwise indicated, the calibration based on PEG standards was used. This technique allowed M_n (the number-average molar mass), M_w (the weight-average molar mass) and M_w/M_n (the polydispersity index, PDI) to be determined. Nanoparticles diameter (D_z) was measured by dynamic light scattering (DLS) with a Nano ZS from Malvern (173° scattering angle) at a temperature of 25 °C. The particle size distribution is generally considered as narrow when below 0.10.

Synthesis of Hexadecyl Cyanoacetate. Hexadecyl cyanoacetate was synthesized as follows. In a 250 mL round-bottom flask containing hexadecane-1-ol (10.65 g, 44 mmol), cyanoacetic acid (7.48 g, 88 mmol), EtOAc (5 mL) and DCM (50 mL) was introduced dropwise by a syringe over ca. 20 min, a solution of DCC (9.98 g, 48.4 mmol) and DMAP (120 mg, 0.82 mmol) in DCM (50 mL). The reaction medium was stirred during 24 h at ambient temperature under argon atmosphere. The solid was filtered off and the solvents were removed under reduced pressure. The solid was then purified by flash chromatography (SiO_2, hexane/EtOAc; 5:1; v:v) to give a fine, white powder: 12.9 g (95%). ^{1}H NMR ($CDCl_3$) δ = 0.88 (t, J = 7.0 Hz, 3H, CH_2CH_3), 1.14−1.50 (m, 26H, CH_2), 1.67 (dd, J = 13.6, 6.8 Hz, 2H, $COOCH_2CH_2$), 3.45 (s, 2H, $CNCH_2$), 4.20 (t, J = 6.8 Hz, 2H, $COOCH_2CH_2$). IR (neat): υ (cm^{-1}) = 2261 (C≡N), 1728 (C═O).

Synthesis of Poly(ethylene glycol) Monobenzyl Ether ($BnPEG_{47}$). $BnPEG_{47}$ was synthesized by ring-opening polymerization of EO as follows. Benzyl alcohol (1.70 g, 16 mmol) and naphthalene potassium (2.68 g, 16 mmol) were added to 250 mL of freshly distilled THF under nitrogen. After 10 min of stirring, the solution was transferred to a 1 L stainless steel reactor and EO (35 g, 0.79 mol) was added to the solution. The polymerization of EO proceeded for 4 h at 50 °C. An excess of diluted hydrochloride aqueous solution was then added to stop the reaction and the polymer was quantitavely recovered by precipitation into a large volume of heptane and dried under vacuum. ^{1}H NMR ($CDCl_3$) δ = 3.35−3.92 (m, 188H, OCH_2CH_2O), 4.53 (s, 2H, $C_6H_5OCH_2$), 7.32 (m, 5H, $C_6H_5OCH_2$). $M_{n,NMR}$ = 2150 g·mol^{-1}, $DP_{n,NMR}$ = 47; $M_{n,SEC}$ = 1920 g·mol^{-1} M_w/M_n = 1.07.

Synthesis of $N_3PEG_{70}CA$ Following Path A. *Synthesis of Benzylpoly(ethylene glycol) Acetate ($BnPEG_{70}OAc$,* **2***).* In a 50 mL round-bottom flask, a solution of $BnPEG_{70}$ (1.0 g, 0.32 mmol), DMAP (30 mg, 0.24 mmol), TEA (124 μL, 0.88 mmol), and acetic anhydride (90 mg, 0.88 mmol) in DCM (15 mL) was allowed to stir at room temperature during 2 h under argon atmosphere. The mixture was then washed three times with 1 M aqueous HCl solution and once with brine. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The solid was dissolved in a minimal amount of DCM and precipitated by dropwise addition in a large volume of cold Et_2O. The product was collected by filtration as a fine, white powder: 993 mg (98%). ^{1}H NMR (D_2O) δ = 2.10 (s, 3H, $OCOCH_3$), 3.40−3.95 (m, 280H, OCH_2CH_2O), 4.24 (t, 2H, J = 4.4 Hz, $COOCH_2$), 4.59 (s, 2H, $C_6H_5OCH_2$), 7.42 (m, 5H, $C_6H_5OCH_2$). IR (neat): υ (cm^{-1}) = 1737 (C═O). $M_{n,SEC}$ = 2750 g·mol^{-1}, M_w/M_n = 1.06.

Synthesis of Poly(ethylene glycol) Acetate ($PEG_{70}OAc$, **3***).* A suspension of **2** (500 mg, 0.156 mmol), EtOH (4 mL), acetic acid (600 mg, 10.0 mmol), and $Pd(OH)_2$/C (80 mg, 0.057 mmol) was hydrogenated at 6 bar in a steel autoclave during 15 h at ambient temperature under vigorous stirring. After the reaction, the catalyst was filtered off and the resulting solution was concentrated under reduced pressure and dried under vacuum to give a slightly yellow solid: 462 mg (95%). ^{1}H NMR (D_2O) δ = 2.11 (s, 3H, $OCOCH_3$), 3.40−3.95 (m, 280H, OCH_2CH_2O), 4.24 (t, 2H, J = 4.4 Hz, $COOCH_2$). IR (neat): υ (cm^{-1}) = 1735 (C═O). $M_{n,SEC}$ = 2690 g·mol^{-1}, M_w/M_n = 1.06.

Synthesis of Methanesulfonylpoly(ethylene glycol) Acetate ($MsPEG_{70}OAc$, **4***).* In a 50 mL round-bottom flask, a solution of **3** (462 mg, 0.148 mmol), DMAP (15 mg, 0.12 mmol), and TEA (90 μL, 0.64 mmol) in DCM (7.6 mL) was cooled to 0 °C. MsCl (40 μL, 0.51 mmol) was then introduced dropwise by a syringe over ca. 15 min. The mixture was then stirred under argon atmosphere at 0 °C during 2 h and overnight at room temperature. The mixture was then washed three times with 1 M aqueous HCl solution and once with brine. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The solid was dissolved in a minimal amount of DCM and precipitated by dropwise addition in a large volume of cold Et_2O. The product was collected by filtration as a fine, white powder: 443 mg (94%). ^{1}H NMR (D_2O) δ = 2.11 (s, 3H, $OCOCH_3$), 3.23 (s, 3H, $CH_2SO_3CH_3$), 3.40−3.95 (m, 280H, OCH_2CH_2O), 4.25 (t, 2H, J = 4.3 Hz, $COOCH_2$), 4.47 (t, 2H, J = 4.3 Hz, $CH_2SO_3CH_3$). IR (neat): υ (cm^{-1}) = 1730 (C═O). $M_{n,SEC}$ = 2700 g·mol^{-1}, M_w/M_n = 1.07.

Synthesis of Azidopoly(ethylene glycol) Acetate ($N_3PEG_{70}OAc$, **5***).* In a 100 mL round-bottom flask equipped with a condenser and an argon inlet was introduced a solution of **4** (250 mg, 0.078 mmol) in DMF (5 mL). NaN_3 (27 mg, 0.41 mmol) was then added and the mixture was stirred during 24 h at 50 °C. DMF was removed under vacuum and a minimum of acetone was added which allowed the excess NaN_3 to be filtered off. Acetone was then removed under reduced pressure. The solid was then dissolved in DCM and the mixture was washed three times with water. The organic phase was dried over $MgSO_4$, filtered, concentrated under reduced pressure and dried under vacuum to give a slightly yellow powder: 238 mg (96%). ^{1}H NMR ($CDCl_3$) δ = 2.08 (s, 3H, $OCOCH_3$), 3.40 (t, 2H, J = 4.5 Hz, CH_2N_3), 3.35−3.92 (m, 280H, OCH_2CH_2O), 4.22 (t, 2H, J = 4.5 Hz, $COOCH_2$). IR (neat): υ (cm^{-1}) = 1738 (C═O), 2104 (N_3). $M_{n,SEC}$ = 2720 g·mol^{-1}, M_w/M_n = 1.06.

Synthesis of Azidopoly(ethylene glycol) (N_3PEG_{70}, **6***).* In a 50 mL round-bottom flask was introduced a solution of **5** (125 mg, 0.04 mmol) in THF (4 mL) and MeOH (6.25 mL). A solution of NaOH (75 mg, 1.9 mmol) in water (0.3 mL) was then added and the mixture was stirred during 18 h at room temperature under argon atmosphere. Then, pH was adjusted to 6 by means of 1 M HCl solution and solvents were evaporated under reduced pressure. The obtained solid was taken into DCM and the resulting organic layer was washed three times with water. The organic phase was then dried over $MgSO_4$, filtered, concentrated under reduced pressure and dried under vacuum to give a slightly yellow powder: 121 mg (98%). ^{1}H NMR ($CDCl_3$) δ = 3.40 (t, 2H, J = 4.5 Hz, CH_2N_3), 3.35−3.92 (m, 280H, OCH_2CH_2O). IR (neat): υ (cm^{-1}) = 2103 (N_3). $M_{n,SEC}$ = 2700 g·mol^{-1}, M_w/M_n = 1.07.

Synthesis of Azidopoly(ethylene glycol) Cyanoacetate ($N_3PEG_{70}CA$, **7b***).* In a 50 mL round-bottom flask containing **6** (90 mg, 0.029 mmol), cyanoacetic acid (46 mg, 0.053 mmol) and DCM (2 mL) was introduced dropwise by a syringe over ca. 15 min, a solution

of DCC (11 mg, 0.053 mmol) and DMAP (15 mg, 0.10 mmol) in DCM (1 mL). The reaction medium was stirred during 24 h at ambient temperature under argon atmosphere. The solid was filtered off and the solvent was removed under reduced pressure. The solid was then purified by recrystallization from isopropanol, filtered and dried under vacuum overnight to give a slightly yellow powder: 86 mg (94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.38 (t, 2H, J = 4.5 Hz, CH_2N_3), 3.54 (s, 2H, $CNCH_2$), 3.35−3.92 (m, 280H, OCH_2CH_2O), 3.73 (t, 2H, J = 4.5 Hz, $COOCH_2CH_2$), 4.35 (t, 2H, J = 4.5 Hz, $COOCH_2CH_2$). ^{13}C NMR ($CDCl_3$) δ = 24.7 ($CNCH_2$), 50.8 (CH_2N_3), 65.7 ($COOCH_2CH_2$), 68.5 ($COOCH_2CH_2$), 70.5 (OCH_2CH_2O), 113.1 (CH_2CN), 163.3 ($COOCH_2$). IR (neat): υ (cm^{-1}) = 2255 (C≡N), 2098 (N_3), 1738 (C═O). $M_{n,SEC}$ = 2750 g·mol^{-1}, M_w/M_n = 1.07.

Synthesis of $N_3PEG_{47}CA$ Following Path B. *Synthesis of Benzylpoly(ethylene glycol) Methanesulfonyl ($BnPEG_{47}Ms$,* **8***).* In a 50 mL round-bottom flask, a solution of $BnPEG_{47}$ (502 mg, 0.23 mmol), DMAP (15 mg, 0.12 mmol) and TEA (180 μL, 1.28 mmol) in DCM (8 mL) was cooled to 0 °C. MsCl (80 μL, 1.02 mmol) was then introduced dropwise by a syringe over ca. 15 min. The mixture was then stirred under argon atmosphere at 0 °C during 2 h and overnight at ambient temperature. The mixture was then washed three times with 1 M aqueous HCl solution and once with brine. The organic phase was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The solid was dissolved in a minimal amount of DCM and precipitated by dropwise addition in a large volume of cold Et_2O. The product was collected by filtration as a fine, white powder: 500 mg (96%). ^{1}H NMR ($CDCl_3$) δ = 3.08 (s, 3H, $CH_2SO_3CH_3$), 3.35−3.92 (m, 188H, OCH_2CH_2O), 4.37 (t, 2H, J = 4.4 Hz, $CH_2SO_3CH_3$), 4.56 (s, 2H, $C_6H_5OCH_2$), 7.33 (m, 5H, $C_6H_5OCH_2$). $M_{n,SEC}$ = 1960 g·mol^{-1}, M_w/M_n = 1.07.

Synthesis of Benzylpoly(ethylene glycol) Azide ($BnPEG_{47}N_3$, **9***).* In a 100 mL round-bottom flask equipped with a condenser and an argon inlet was introduced a solution of **8** (2.32 g, 1.03 mmol) in DMF (20 mL). NaN_3 (0.36 g, 5.43 mmol) was then added and the mixture was stirred during 24 h a 50 °C. DMF was removed under vacuum and a minimum of acetone was added which allowed the excess NaN_3 to be filtered off. Acetone was then removed under reduced pressure. The solid was then dissolved in DCM and the mixture was washed three times with water. The organic phase was dried over $MgSO_4$, filtered, concentrated under reduced pressure and dried under vacuum to give a slightly yellow powder: 2.04 g (91%). ^{1}H NMR ($CDCl_3$) δ = 3.39 (t, 2H, J = 4.5 Hz, CH_2N_3), 3.42−3.93 (m, 188H, OCH_2CH_2O), 4.56 (s, 2H, $C_6H_5OCH_2$), 7.33 (m, 5H, $C_6H_5OCH_2$). IR (neat): υ (cm^{-1}) = 2102 (N_3). $M_{n,SEC}$ = 1950 g·mol^{-1}, M_w/M_n = 1.06.

Synthesis of Azidopoly(ethylene glycol) (N_3PEG_{47}, **10***).* In a 100 mL round-bottom flask, **9** (2.04 g, 0.93 mmol) was dissolved in concentrated aqueous HCl solution and stirred under argon during 48 h at ambient temperature. The mixture was then diluted with 250 mL of water and the pH adjusted at ∼2 with 1 M NaOH aqueous solution. The resulting aqueous layer was extracted with four portions of DCM and the organic layer was collected, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The solid was dissolved in a minimal amount of DCM and precipitated by dropwise addition in a large volume of cold Et_2O. The product was collected by filtration as a fine, white powder: 1.65 g (84%). ^{1}H NMR ($CDCl_3$) δ = 3.40 (t, 2H, J = 4.5 Hz, CH_2N_3), 3.42−3.95 (m, 188H, OCH_2CH_2O). IR (neat): υ (cm^{-1}) = 2104 (N_3). $M_{n,SEC}$ = 1900 g·mol^{-1}, M_w/M_n = 1.07.

Synthesis of Azidopoly(ethylene glycol) Cyanoacetate ($N_3PEG_{47}CA$, **7a***).* In a 50 mL round-bottom flask containing **10** (1.60 g, 0.76 mmol), cyanoacetic acid (0.14 g, 1.61 mmol) and DCM (7 g) was introduced dropwise by a syringe over ca. 15 min a solution of DCC (330 mg, 1.60 mmol) and DMAP (60 mg, 0.41 mmol) in DCM (1 g). The reaction medium was stirred during 24 h at ambient temperature under argon atmosphere. The solid was filtered off and the solvent was removed under reduced pressure. The solid was then purified by recrystallization from isopropanol, filtered and dried under vacuum overnight to give a slightly yellow powder: 1.58 g (96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.38 (t, 2H, J = 4.5 Hz, CH_2N_3), 3.54 (s, 2H, $CNCH_2$), 3.55−3.70 (m, 188H, OCH_2CH_2O), 3.73 (t, 2H, J = 4.5 Hz, $COOCH_2CH_2$), 4.35 (t, 2H, J = 4.5 Hz, $COOCH_2CH_2$). ^{13}C NMR ($CDCl_3$) δ = 24.7 ($CNCH_2$), 50.7 (CH_2N_3), 65.8 ($COOCH_2CH_2$), 68.5 ($COOCH_2CH_2$), 70.6 (OCH_2CH_2O), 113.2 ($CNCH_2$), 163.3 ($COOCH_2$). IR (neat): υ (cm^{-1}) = 2261 (C≡N), 2102 (N_3), 1739 (C═O). $M_{n,SEC}$ = 1920 g·mol^{-1}, M_w/M_n = 1.08.

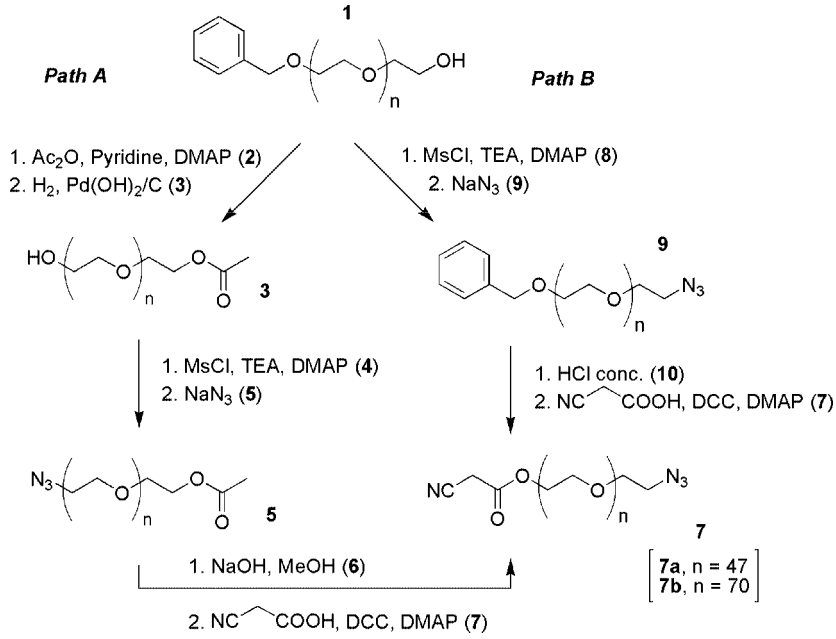
The synthesis of $N_3PEG_{70}CA$ (**7b**) was also achieved from $BnPEG_{70}$ following Path B with similar intermediate yields (overall yield 68%) and identical ^{1}H, ^{13}C NMR and IR results. $M_{n,SEC}$ = 2690 g·mol^{-1}, M_w/M_n = 1.07.

Synthesis of Methoxypoly(ethylene glycol) Cyanoacetate ($MePEG_{43}CA$). $MePEG_{43}CA$ was synthesized as follows. In a 100 mL round-bottom flask containing poly(ethylene glycol) monomethyl ether (11.0 g, DP_n = 45, 5.5 mmol), cyanoacetic acid (0.955 g, 11.0 mmol) and DCM (30 mL) was introduced dropwise by a syringe over ca. 20 min, a solution of DCC (2.27 g, 11.0 mmol) and DMAP (60 mg, 0.41 mmol) in DCM (10 mL). The reaction medium was stirred during 24 h at room temperature under argon atmosphere. The solid was filtered off and the solvent was removed under reduced pressure. The solid was then purified by recrystallization from isopropanol, filtered and dried under vacuum overnight to give a fine, white powder: 10.7 g (94%). ^{1}H NMR ($CDCl_3$) δ = 3.34 (s, 3H, OCH_3), 3.53 (s, 2H, $CNCH_2$), 3.25−3.92 (m, 172H, OCH_2CH_2O), 4.32 (t, 2H, J = 4.5 Hz, $COOCH_2CH_2$). IR (neat): υ (cm^{-1}) = 1745 (C═O), 2251 (C≡N). $M_{n,SEC}$ = 1890 g·mol^{-1}, M_w/M_n = 1.04.

Synthesis of Poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-$N_3PEG_{47}CA$)) Copolymer. The P(HDCA-*co*-$N_3PEG_{47}CA$) copolymer was prepared as follows. In a 50 mL round-bottom flask containing **7a** (n = 47, 1.0 g, 0.48 mmol), HDCA (0.6 g, 1.9 mmol), EtOH (5 mL) and DCM (10 mL), under magnetic stirring, were sequentially introduced dropwise by a syringe, over ca. 20 min, formalin (1 mL, 13.3 mmol) and pyrrolidine (50 μL, 0.61 mmol). The mixture was allowed to stir during 24 h at room temperature and was then concentrated under reduced pressure. The residue was taken into DCM and washed three times with water, one time with 1 M aqueous HCl solution and once with brine. The resulting organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure and dried under vacuum to give a slightly yellow powder: 1.55 g. The same procedure was used with compound **7b** (n = 70) to achieve the P(HDCA-*co*-$N_3PEG_{70}CA$) copolymer. Copolymers were analyzed by ^{1}H NMR (Figure 3) and IR spectroscopy (see text). From a calibration based on PMMA standards, SEC gave: for the P(HDCA-*co*-$N_3PEG_{47}CA$) copolymer, $M_{n,SEC}$ = 2000 g·mol^{-1} and M_w/M_n = 1.82; for the P(HDCA-*co*-$N_3PEG_{70}CA$) copolymer, $M_{n,SEC}$ = 2800 g·mol^{-1} and M_w/M_n = 2.0.

Click Reaction between 4-Pentyn-1-ol and Poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-$N_3PEG_{70}CA$)) Copolymer in Organic Medium. A typical click reaction in organic medium using P(HDCA-*co*-$N_3PEG_{70}CA$) copolymer obtained from compound **7b** (n = 70) was as follows. A solution of P(HDCA-*co*-$N_3PEG_{70}CA$) copolymer (23 mg, 4.96 μmol) and 4-pentyn-1-ol (4.3 mg, 50 μmol) in DMF (1 mL) was placed in a 5 mL round-bottom flask sealed with a rubber septum and degassed by argon bubbling during 30 min. Then, CuBr (1.5 mg, 10 μmol) was added to the reaction medium and argon bubbling was continued for further 10 min. PMDETA (3.6 mg, 4.3 μL, 20 μmol) was then introduced by a syringe and the reaction medium turned dark purple. The resulting solution was allowed to stir during 6 h at room temperature under argon atmosphere. At the end of the reaction, the rubber septum was removed and the solution was diluted with 1 mL of DMF under stirring, leading to oxidation of Cu(I) catalyst into Cu(II). Then, the catalyst was removed by passing the organic solution through a basic alumina column, and the solvent was evaporated under vacuum. The same protocol was followed with the P(HDCA-*co*-$N_3PEG_{47}CA$) copolymer obtained from compound **7a** (n = 47). The resulting copolymers were analyzed by ^{1}H NMR (Figure 5) and IR spectroscopy

Scheme 2. Synthetic Pathways to Prepare Azidopoly(ethylene glycol) Cyanoacetate (N_3PEGCA, 7) from Benzyl−PEG (BnPEG, 1, n = 47 or 70): DMAP = 4-Dimethylaminopyridine; TEA = Triethylamine; DCC = *N*,*N*-Dicyclohexylcarbodiimide



(see text). From a calibration based on PMMA standards, SEC gave: for the P(HDCA-*co*-N_3PEG$_{47}$CA) copolymer, $M_{n,SEC}$ = 2110 g·mol^{-1} and M_w/M_n = 1.95; for the P(HDCA-*co*-N_3PEG$_{70}$CA) copolymer, $M_{n,SEC}$ = 2860 g·mol^{-1} and M_w/M_n = 2.1.

Synthesis of Pent-4-ynoic-methoxypoly(ethylene glycol) Ester (Alkyne−PEG$_{43}$). In a 50 mL round-bottom flask containing poly(ethylene glycol) monomethyl ether (5.0 g, 2.5 mmol), 4-pentynoic acid (0.5 g, 5.0 mmol) and DCM (30 mL) was introduced dropwise by a syringe over ca. 20 min, a solution of DCC (1.03 g, 4.99 mmol) and DMAP (50 mg, 0.41 mmol) in DCM (10 mL). The reaction medium was stirred during 24 h at ambient temperature under argon atmosphere. The solid was filtered off and the solvent was removed under reduced pressure. The solid was then purified by recrystallization from isopropanol, filtered and dried under vacuum overnight to give a fine, white powder: 4.8 g (92%). ^{1}H NMR ($CDCl_3$) δ = 1.98 (s, 1H, C≡C*H*), 2.45−2.65 (m, 4H, OOCCH_2CH_2), 3.37 (s, 3H, OCH_3), 3.32−3.92 (m, 172H, OCH_2CH_2O), 4.26 (t, 2H, J = 4.5 Hz, COOCH_2CH_2). ^{13}C NMR ($CDCl_3$) δ = 14.3 (CH_2CH_2C≡CH), 33.2 (CH_2CH_2C≡CH), 59.0 (OCH_3), 63.8 (CH_2OOCCH_2), 69.0 (CH_2CH_2OOC), 69.1 (C≡CH),

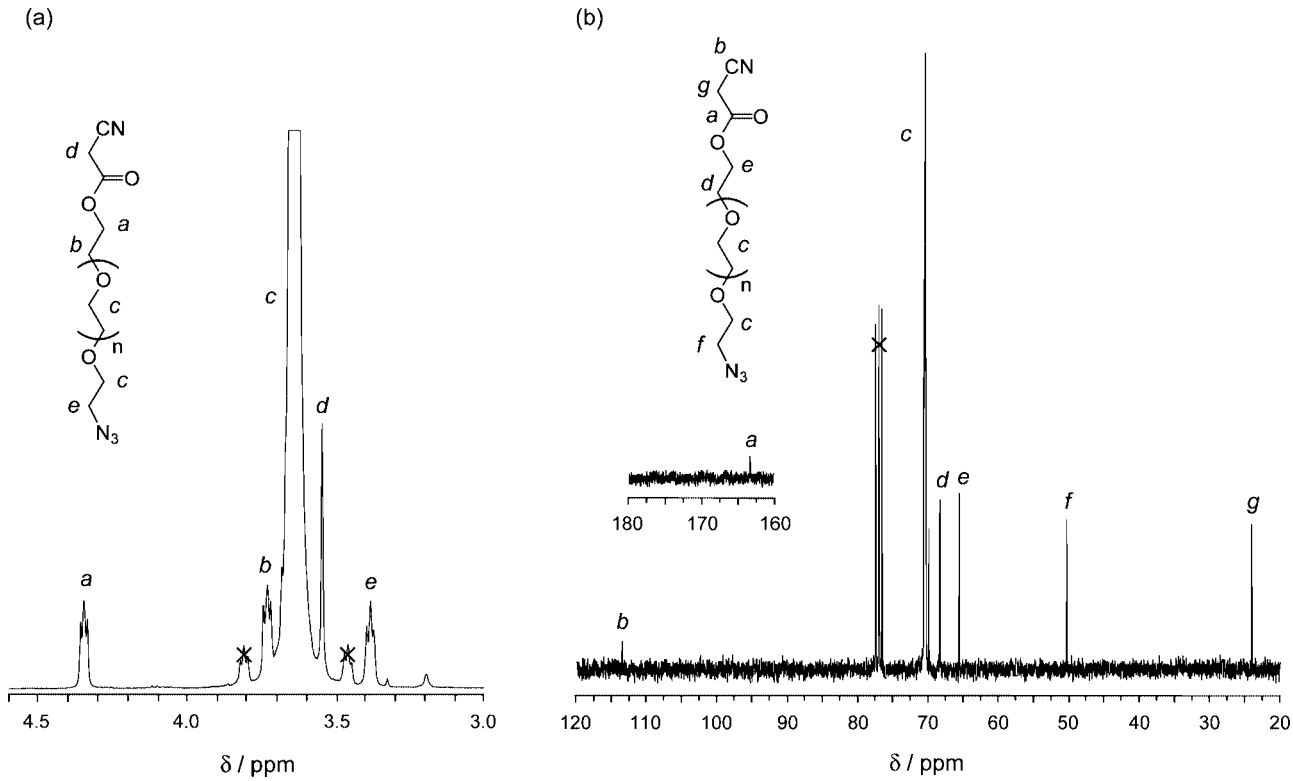


Figure 2. 400 MHz ^{1}H (a) and 75 MHz ^{13}C (b) NMR spectra in $CDCl_3$ of the azidopoly(ethylene glycol) cyanoacetate (N_3PEG$_{47}$CA, **7a**) synthesized following Path A (see Scheme 2).

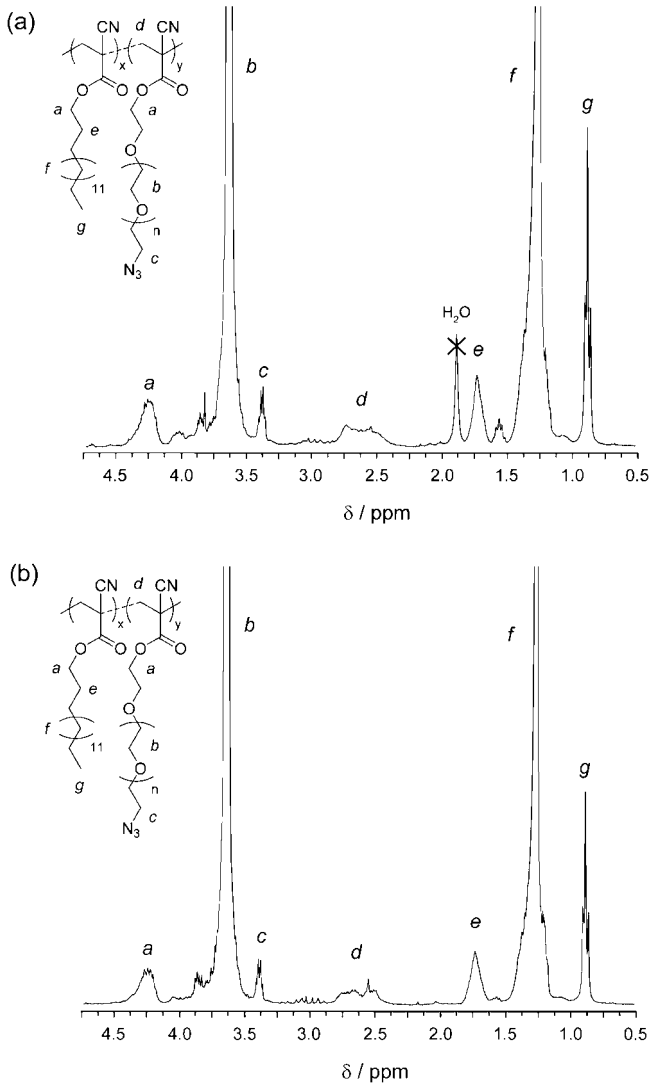


Figure 3. 300 MHz ^{1}H NMR spectrum in $CDCl_3$ of the poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-N_3PEG_nCA)) copolymer; $n = 47$ (a) and 70 (b).

70.6 (OCH_2CH_2O), 71.9 (CH_2OCH_3), 82.4 ($C{\equiv}CH$), 171.7 ($COOCH_2$). IR (neat): υ (cm^{-1}) = 2187 (C≡C), 1737 (C═O). $M_{n,SEC} = 1970$ g·mol^{-1}, $M_w/M_n = 1.04$.

Preparation of P(HDCA-*co*-N_3PEG_nCA) Nanoparticles. Nanoparticles were prepared by the nanoprecipitation technique.[16] In practice, the P(HDCA-*co*-N_3PEG_nCA) copolymer (30 mg) was dissolved in acetone (6 mL), and the copolymer solution was added dropwise, under mechanical stirring, to deionized water (12 mL). A milky suspension was observed almost instantaneously. Acetone was then evaporated under reduced pressure to yield a stable P(HDCA-*co*-N_3PEG_nCA) copolymer suspension of 2.5 mg.mL^{-1} (see Table 1 for colloidal characteristics of the nanoparticles).

Click Reaction between Alkyne−PEG_{43} and Poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-N_3PEG_{70}CA)) Copolymer in Aqueous Dispersed Medium. A typical click reaction in aqueous dispersed medium using P(HDCA-*co*-N_3PEG_{70}CA) copolymer obtained from compound **7b** ($n = 70$) was as follows. To a stable suspension of P(HDCA-*co*-N_3PEG_{70}CA) copolymer obtained immediately after nanoprecipitation (i.e., without removing acetone), was sequentially added, alkyne-PEG_{43} (180 mg, 8.8 μmol) and a freshly prepared solution of sodium ascorbate (1.75 mg, 8.8 μmol) and $CuSO_4.5H_2O$ (1.1 mg, 4.4 μmol). The reaction mixture was allowed to stir at ambient temperature during 70 h under argon atmosphere. Then, the nanoparticles suspension was extensively dialyzed (6000−8000 MWCO) against a 1 M NaCl aqueous solution to remove the copper catalyst and the alkyne-PEG in excess and then against deionized water. The copolymer was then recovered by rotary evaporation as a white solid and was analyzed by ^{1}H NMR spectroscopy (Figure 7) and SEC (Figure 8).

Results and Discussion

1. Synthesis of Clickable PACA Copolymer. The general methodology we propose herein consists in synthesizing a PACA-PEG biodegradable copolymer, via tandem Knoevenagel condensation−Michael addition reaction between the corresponding cyanoacetates, displaying azido groups at the extremity of PEG chains for further click reaction with alkyne derivatives. To do so, our strategy was to prepare N_3PEGCA heterobifunctional PEGs, from different synthetic pathways, both starting from benzyl−PEG (BnPEG) of variable chain lengths (DP_n = 47 or 70). First, benzylpoly(ethylene glycol) cyanoacetate ($BnPEG_{70}CA$) was prepared by direct coupling of $BnPEG_{70}$ with cyanoacetic acid, followed by the deprotection of the benzyl group via catalytic hydrogenation using $Pd(OH)_2$/C. Unfortunately, concomitantly to the removal of the benzyl group, a significant reduction of the cyano function was noticed by ^{1}H NMR spectroscopy. Therefore, more efficient protocols were investigated based on the early deprotection of the benzyl group with respect to the coupling reaction with cyanoacetic acid, together with nearly quantitative reactions and very simple purification workups (Scheme 2). In the first protocol (Scheme 2, Path A), the hydroxyl group was first protected before catalytic hydrogenation of the benzyl function (**3**). Then, the recovered hydroxyl group was readily converted into an azide functionality (**5**) by consecutive treatment with methanesulfonyl chloride and sodium azide. Eventually, the acetyl group was removed followed by coupling with cyanoacetic acid to yield the desired N_3PEGCA (**7**) with a very good overall yield (77%). For the second protocol (Scheme 2, Path B), which required less steps, the hydroxyl group of BnPEG was converted into an azide functionality (**9**) before removal of the benzyl group by treatment with concentrated hydrochloric acid,[72] without any alteration of the PEG chain or the azide moiety (**10**). Then, coupling reaction with cyanoacetic acid was undertaken to yield the N_3PEGCA moiety (**7**) with good overall yields (68−70%).

For both pathways, all intermediates recovered after each steps were pure (see experimental part) and the final N_3PEGCA exhibited the expected ^{1}H and ^{13}C NMR signals, no matter the molar mass of the starting BnPEG (Figure 2). In particular, the presence of the azide functionality at the end of the PEGCA chain was clearly demonstrated: the peak *e* at 3.38 ppm on the ^{1}H NMR spectrum (Figure 2a) was assigned to the methylene in the α-position of the azide group whereas the peak *f* at 50.56 ppm on the ^{13}C NMR spectrum (Figure 2b) was attributed to the carbon in the α-position of the azide group. It is worth mentioning that the coupling reaction with cyanoacetic acid was also demonstrated by the peaks *d* and *g* on the ^{1}H and ^{13}C NMR spectra, respectively, assigned to the methylene in the α-position of the cyano group. From a quantitative standpoint, integration data showed an excellent correlation between each expected and calculated numbers of protons. Size exclusion chromatography analysis of N_3PEG_nCA moieties (n = 47 or 70) gave a monomodal peak identical to the parent BnPEG, together with no traces of side-product and/or altered PEG (see experimental part for SEC data).

The synthesis of P(HDCA-*co*-N_3PEG_nCA) copolymers was then achieved from HDCA and N_3PEG_nCA (**7**, n = 47 or 70), with a 4.0:1 initial molar ratio via Knoevenagel condensation−Michael addition reaction. The ^{1}H NMR spectra of the resulting materials were consistent with the expected structure of the copolymers (Figure 3) and also in very good agreement with

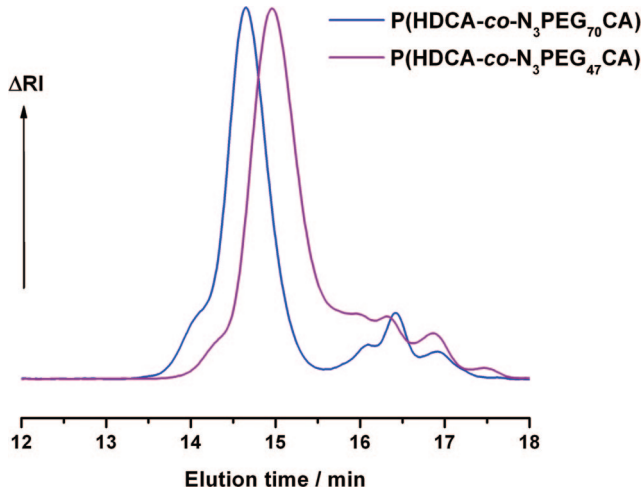


Figure 4. Size exclusion chromatography analysis of poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-N_3PEGCA)) copolymers obtained by Knoevenagel condensation−Michael addition reaction between hexadecyl cyanoacetate (HDCA) and azidopoly(ethylene glycol) cyanoacetate (N_3PEG_nCA; n = 47 or 70).

previous results concerning P(HDCA-*co*-MePEGCA) copolymers.[28] The formation of the polymeric backbone was identified by the broad signal at 2.3−2.8 ppm, endorsed by the broad peak at 4.2−4.4 ppm assigned to the methylene in the α-position of the ester function involved in the macromolecular structure. Considering the average copolymer compositions, integration of peaks *b* and *f* gave a HDCA:N_3PEG_nCA molar ratio of 4.2:1 (n = 47) and 4.1:1 (n = 70), very close of the initial stoichiometry. Even if overlaid with one of the PEG satellite, the peak *c* at 3.4 ppm was assigned to the methylene in α-position of the azide function, the signal of which was also detected by IR spectroscopy at 2103 cm^{-1}.

It has been previously shown[73] by near-infrared measurements that Knoevenagel condensation−Michael addition reaction between HDCA and PEGCA derivatives does not lead to well-defined P(HDCA-*co*-PEGCA) random copolymers, but to a complex mixture of various oligomers exhibiting different amphiphilic properties, probably due to a difference of steric hindrance between the starting cyanoacetates. The size exclusion chromatograms of the above-mentioned copolymers totally confirmed those early observations (Figure 4). Indeed, whereas a significant tailing toward lower molar masses was noticed on the chromatogram of the P(HDCA-*co*-$N_3PEG_{47}CA$) copolymer, a detectable amount of PHDCA homopolymer (which elutes earlier than HDCA) was visible when the P(HDCA-*co*-$N_3PEG_{70}CA$) copolymer was analyzed. This low molecular weight polymer might arise either from Knoevenagel-Michael addition homocondensation reaction between HDCA moieties and/or from a rapid depolymerisation of the (co)polymer accompanied by simultaneous repolymerization of the resulting monomer to yield lower molecular weight species, as often observed with poly(alkyl cyanoacrylate).[74]

Nevertheless, even though these intrinsic features of alkyl cyanoacrylate chemistry might be considered as a drawback regarding polymer synthesis, note that during nanoparticles formation by self-assembly in aqueous medium, PHDCA homopolymer is entrapped into the core of the nanoparticles stabilized by P(HDCA-*co*-PEGCA) copolymers to eventually lead to well-defined colloidal objects. Besides, to the best of our knowledge, this is the only method available to yield biodegradable PACA nanoparticles covered by PEG chains in mild conditions (as opposed to direct emulsion polymerization of alkyl cyanoacrylates in strong aqueous acidic media). For the sake of simplicity, the term copolymer will be employed in the text to refer to the oligomers mixture resulting from Knoevenagel condensation−Michael addition reaction.

2. Click Reaction in Homogeneous Medium. The clicking ability of the P(HDCA-*co*-N_3PEG_nCA) copolymer was first investigated in homogeneous medium (DMF) using CuBr:PMDETA as the catalyst and 4-pentyn-1-ol as a small model molecule. Under mild experimental conditions, both P(HDCA-*co*-N_3PEG_nCA) copolymers (n = 47 and 70) were efficiently clicked with 4-pentyn-1-ol in a quantitative fashion. Indeed, IR spectroscopy of the final clicked copolymers showed a total disappearance of the azide signal at 2103 cm^{-1}. Besides, ^1H

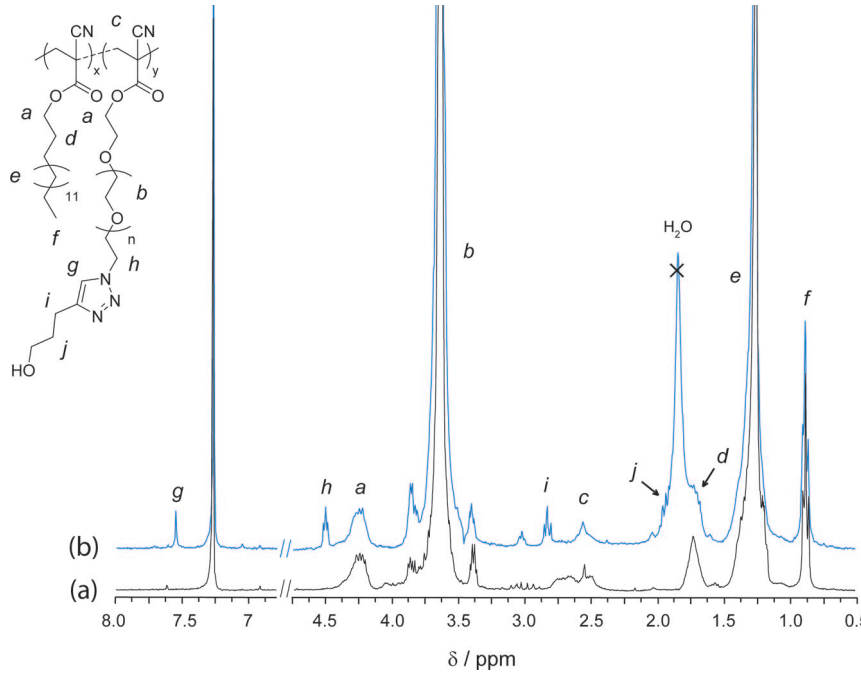


Figure 5. 300 MHz ^1H NMR spectrum in $CDCl_3$ of the poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-$N_3PEG_{70}CA$)) copolymer before (a) and after (b) click reaction with 4-pentyn-1-ol in *N*,*N*-dimethylformamide.

Table 1. Colloidal Characteristics of Poly[(hexadecyl cyanoacrylate)-*co*-methoxypoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-MePEGCA)) and Poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-N_3PEG_nCA)) Nanoparticles before and after Click Reactions

	RPEG$_n$CA				
entry	R	*n*	click reaction alkyne (solvent)	av particle diameter (D_z) (nm)	particle size distribution
1	OMe	43		99[a]	0.073
2	N_3	47		105[a]	0.073
3	N_3	47	4-pentyn-1-ol (*N*,*N*-dimethylformamide)	106[a]	0.068
4	N_3	47	alkyne-dansyl (*N*,*N*-dimethylformamide)	95[a]	0.098
5	N_3	70		82[a]	0.094
6	N_3	70	alkyne-PEG (H_2O/acetone)	95[b]	0.069

[a] Measurement performed after nanoprecipitation without any further purification. [b] Measurement performed after dialysis.

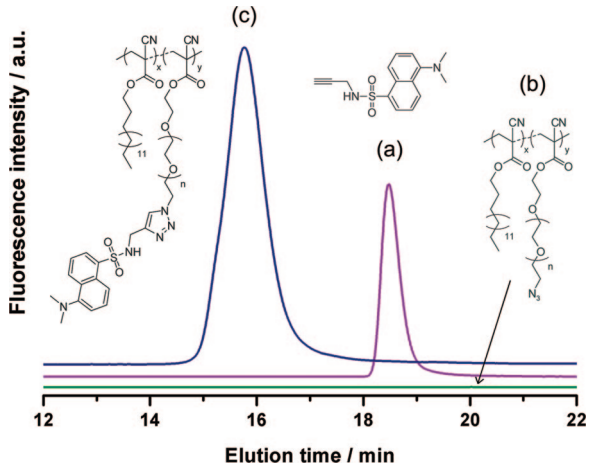


Figure 6. Size exclusion chromatography analysis with a fluorescence detector ($\lambda_{ex.}$ = 340 nm; $\lambda_{em.}$ = 520 nm) of alkyne−dansyl (a), poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-N_3PEG_{47}CA)) copolymer before (b) and after click reaction with alkyne-dansyl in *N*,*N*-dimethylformamide followed by dialysis (c).

NMR spectroscopy showed all expected peaks accounting for the formation of the triazole ring (Figure 5). For instance, peak *g* and peak *h* were respectively assigned to the triazole ring proton and to the α-methylene. Integration data using peak *h* and peak *b* gave a coupling efficiency of 93% (n = 47) and 95% (n = 70), which demonstrated that azide moieties have been almost quantitavely clicked, whatever the PEG chain length (also suggesting that azide groups were not altered by the copolymerization process, thus remaining available for further coupling reactions). Furthermore, SEC did not show any traces of cross-linked materials resulting from a possible coupling reaction between nitrile groups of the copolymer and the azide end groups[75] (which is not surprising since experimental conditions to achieve such a reaction are very harsh compared to the classical click conditions employed here).

Stable nanoparticles from the starting P(HDCA-*co*-N_3PEG_{47}-CA) and from the clicked copolymer were then prepared by the nanoprecipitation technique and analyzed by DLS (Table 1). From these measurements, azide-functionalized nanoparticles exhibited an average diameter very close to nearly identical P(HDCA-*co*-$MePEG_{43}$CA) nanoparticles (entries 1 and 2, Table 1). Besides, it was satisfying to see that upon clicking, particle size distribution remained narrow with no significant difference

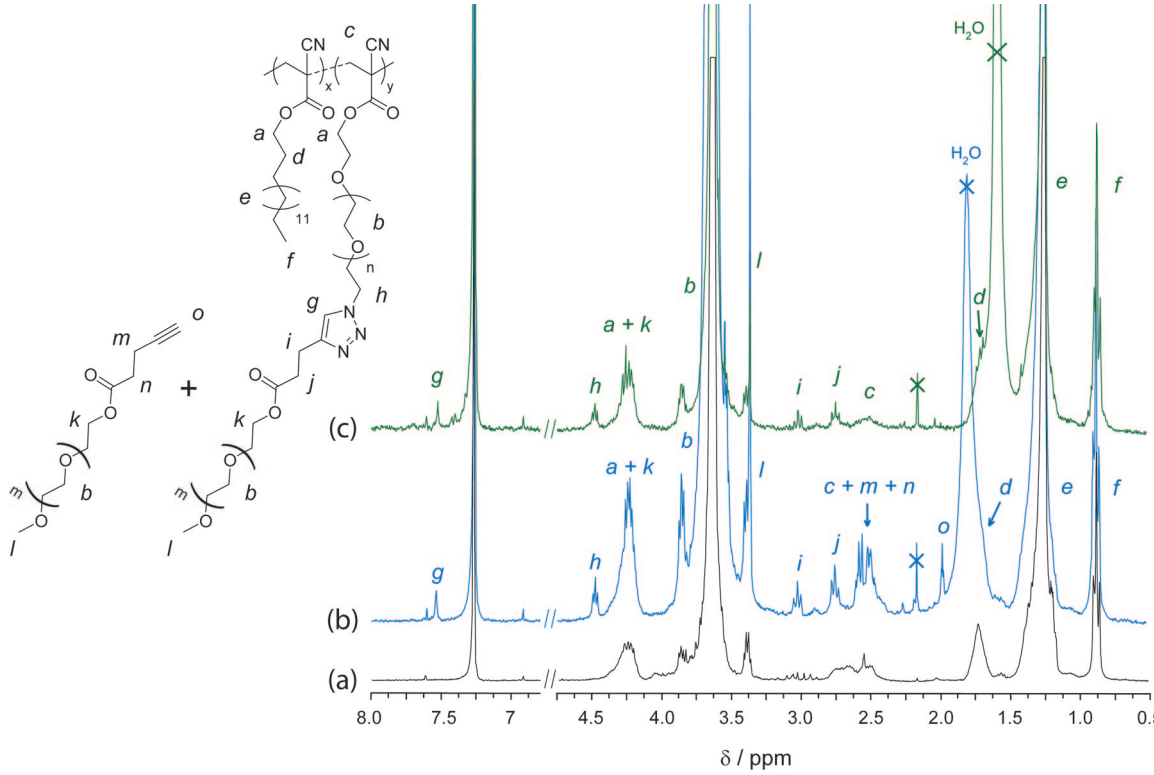


Figure 7. 300 MHz ^{1}H NMR spectrum in $CDCl_3$ of the poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-N_3PEG_{70}CA)) nanoparticles before (a), after the click reaction with alkyne-PEG_{43} in aqueous dispersed medium (b), and after dialysis (c).

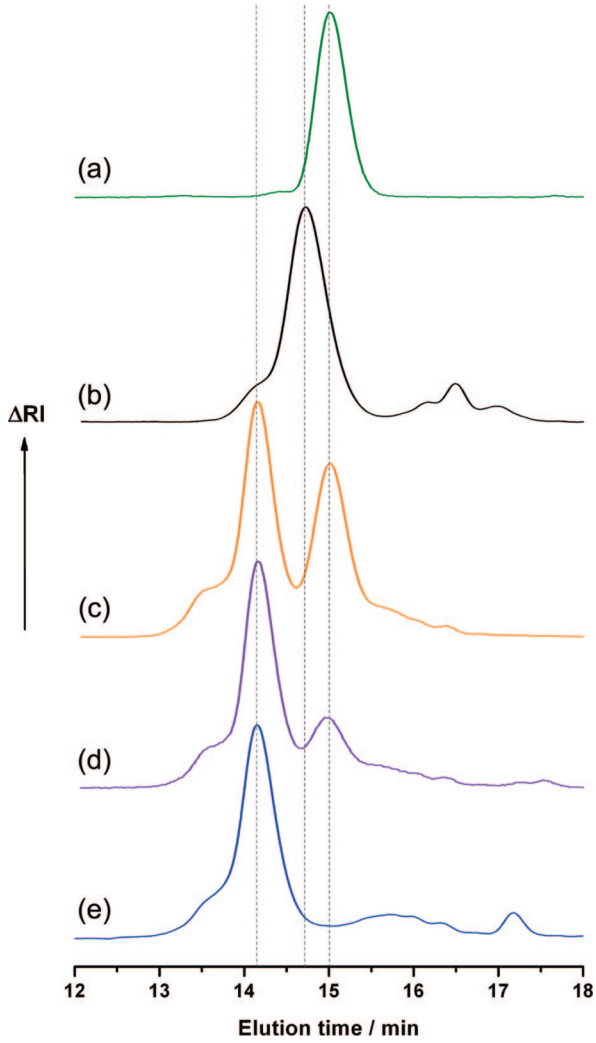


Figure 8. Size exclusion chromatography analysis of alkyne-PEG_{43} (a), poly[(hexadecyl cyanoacrylate)-*co*-azidopoly(ethylene glycol) cyanoacrylate] (P(HDCA-*co*-$N_3PEG_{70}CA$)) copolymer before (b) and after click reaction with alkyne−PEG_{43} in aqueous dispersed medium at various dialysis times (c−e).

in average diameter, accounting for no alteration of the copolymer amphiphilic properties (entries 2 and 3, Table 1).

After having demonstrated the feasibility of the approach in homogeneous medium, these results open the door to the efficient conjugation of small, biorelated compounds, directly to the P(HDCA-*co*-N_3PEG_nCA) copolymer, followed by self-assembly in aqueous solution and resulting in highly functionalized PACA nanoparticles. In this view, the idea was to click a fluorescent probe on the P(HDCA-*co*-N_3PEG_nCA) copolymer in order to prepare fluorescent nanoparticles based on poly(alkyl cyanoacrylate). Materials containing fluorescent tags are indeed crucial for tracing in biological systems during biomedical assays as the location of the material can be finely observed. An alkyne−dansyl was synthesized[70] and successfully clicked on the P(HDCA-*co*-$N_3PEG_{70}CA$) copolymer. After removal of the catalyst, well-defined nanoparticles were obtained by self-assembly in aqueous medium (see entry 4, Table 1) followed by extensive dialysis against a 1:2 acetone/buffered water (sodium chloride 1 M, sodium phosphate 50 mM) mixture, in order to efficiently remove the unreacted dansyl probe before subsequent lyophilization. From SEC analysis with a fluorescence detector (Figure 6), it was observed that no remaining alkyne-dansyl was detected on the purified materials and, as expected, the clicked copolymer, which eluted at higher elution time, $t_{el.}$, exhibited a strong fluorescence intensity (at appropriate $\lambda_{ex.}$ and $\lambda_{em.}$), in contrast to the starting P(HDCA-*co*-$N_3PEG_{70}CA$) copolymer where no signal was detected. This confirmed that the fluorescent probe was well-incorporated into the copolymer structure, via the azidopoly(ethylene glycol) moieties.

3. Click Reaction in Aqueous Dispersed Medium. The next step of our study was to investigate the coupling reaction at the surface of the P(HDCA-*co*-N_3PEGCA) nanoparticles, acting here as a clickable colloidal scaffold (Figure 1b). This pathway appears here as the best choice when nanoparticles have to be functionalized with large molecules (peptides, proteins, etc.). In order to mimic such a water-soluble ligand, an alkyne−PEG has been prepared and used as a model alkyne for click reaction with azido-decorated PACA nanoparticles. The alkyne-PEG_{43} was synthesized from commercially available poly(ethylene glycol) monomethyl ether ($M_{n,SEC}$ = 1970 g·mol^{-1}) and 4-pentynoic acid via standard DCC coupling protocol in very good yield (see experimental part). Once the P(HDCA-*co*-$N_3PEG_{70}CA$) nanoparticles were formed by nanoprecipitation, alkyne-PEG_{43} in excess and a $CuSO_4.5H_2O$/sodium ascorbate mixture were added to the colloidal suspension. After the reaction, the copper catalyst and the alkyne-PEG in excess were removed by dialysis. From ^{1}H NMR spectroscopy of the lyophilized nanoparticles in $CDCl_3$, characteristic peaks demonstrating the successful cycloaddition between alkyne−PEG_{43} and the azido−PEG chains were noticed (Figure 7). Indeed, on the spectrum of the final raw mixture (Figure 7b), peaks *g*, *h*, *i* and *j*, belonging respectively to protons on the triazole ring, in α-, α′- and *β*-position of the triazole, were clearly visible. After the dialysis step (Figure 7c), the alkyne−PEG_{43} in excess was efficiently removed as proven by the complete disappearance of peak *o* (acetylenic proton) and peaks $m + n$ (methylene in α- and *β*-positions of the alkyne function), as well as by the significant decrease of the relative intensity of peak $a + k$ (methylene in α-position of the ester function). In a similar way as for click reaction in homogeneous medium, integration data using peaks *h* and $a + k$ (Figure 7c) gave a coupling efficiency of 92% which suggested a nearly quantitative conjugation of the alkyne-PEG_{43} with the P(HDCA-*co*-$N_3PEG_{70}CA$) nanoparticles.

Size exclusion chromatography was also used to confirm the efficiency of the coupling reaction (Figure 8). In addition to its water-solubility, the choice of a model alkyne based on PEG was also governed by the fact that a coupling reaction between two PEGs (in our case, alkyne-PEG_{43} and $N_3PEG_{70}CA$ moiety) would be readily quantified by a SEC calibration based on PEG standards. Upon clicking, a significant shift of the SEC main peak of the P(HDCA-*co*-$N_3PEG_{70}CA$) copolymer toward higher molar mass ($t_{el.}$ = 14.2 min) was observed (Figure 8b and 8c), together with a second peak coming out at $t_{el.}$ = 15.0 min which was assigned to the remaining alkyne-PEG_{43} in excess. From a calibration curve based on PEG standards, this shift toward higher molar mass accounted for 2000 g·mol^{-1}, in excellent agreement with the M_n of the clicked alkyne-PEG_{43}. The remaining alkyne-PEG_{43} was then efficiently removed by dialysis (Figure 8d and 8e) and no residual, unreacted P(HDCA-*co*-$N_3PEG_{70}CA$) copolymer was noticed. These results are a second proof of the successful click reaction occurring in aqueous dispersed medium with the P(HDCA-*co*-$N_3PEG_{70}CA$) nanoparticles. Concerning the colloidal characteristics, DLS gave an average diameter of 95 nm, slightly above the average diameter before the click reaction, but still exhibiting a narrow particle size distribution (entries 5 and 6, Table 1).

Conclusion

For the first time, a general methodology was proposed to prepare highly functionalizable PACA-PEG copolymers and associated nanoparticles. This approach relied on the synthesis of a novel P(HDCA-*co*-N_3PEGCA) copolymer by Knoevenagel condensation−Michael addition reaction, able to efficiently react with alkyne derivatives via Huisgen 1,3-dipolar cyclo-addition, the so-called click chemistry. As a proof of concept, model molecules have been quantitatively coupled either to the P(HDCA-*co*-N_3PEGCA) copolymers in homogeneous medium followed by nanoprecipitation or directly at the surface of the P(HDCA-*co*-N_3PEGCA) nanoparticles in aqueous dispersed medium, acting here as a clickable colloidal scaffold. In all cases, stable clicked nanoparticles were obtained and easily recovered. These results are believed to be of high interest regarding the field of drug delivery as long as they open the door to ligand-functionalized, biodegradable and "stealth" colloidal drug carriers using alkyl cyanoacrylate monomers.

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