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"Click" Reactions: Novel Chemistries for Forming Well-defined Polyester Nanoparticles

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ABSTRACT: We present the synthesis of discrete functionalized polyester nanoparticles in selected nanoscale size dimensions via a controlled intermolecular chain cross-linking process facilitated via "click"-chemistry approaches such as the Cu(I)-catalyzed 1,3-dipolar cycloaddition of azides and thiol−ene reactions. Both "click" reactions led to the formation of well-defined nanoparticles with narrow size distribution and selected nanoscopic size dimensions. The controlled coupling involves the cross-linking of an alkyne functionalized polyester with a bisazide, 1,8-diazide-3,6-dioxaoctane, and an allyl functionalized polyester with 3,6-dioxa-1,8-octanedithiol. The linear functional polyester precursors were synthesized via ring-opening copolymerization of δ-valerolactone and α-propargyl-δ-valerolactone as well as with α-ally-δ-valerolactone, respectively. We found that the nanoparticle formation and the control over the nanoscopic dimension are primarily influenced by the degree of the alkyne or allyl entity implemented in the precursor polymer and the amount of bisazide and dithiol cross-linking reagents. These results underline once more the versatility of the intermolecular chain cross-linking reaction which is not limited to epoxide chemistries but can be extended to "click" reactions to form controlled nanoparticles with comparable efficiencies. This work describes the utilization of alkyne−azide and thiol−ene "click" chemistries to facilitate the controlled assembly and cross-linking of supramolecular 3-D structures in selected nanoscopic ranges.

Introduction

Cu(I)-catalyzed 1,3-dipolar cycloaddition of azides and alkynes named "click" chemistry has emerged as an attractive and promising tool to construct novel polymers with well-defined architectures.[1] The introduction and development of the alkyne−azide click approach has had a transformational impact on applications extending to the preparation of dendrimers,[2−4] synthesis of functional block copolymers,[5] synthesis of uniformly structured hydrogels,[6−8] the preparation of enzyme inhibitors *in situ*,[9] and many others.[10−12] The great success of this process relies on its simplicity, efficiency and selectivity, wide scope applicability regardless of the reagents' molecular complexity and ability to occur under aerobic conditions.[13] "Click" chemistry, however, also encompasses other well-known reactions, such as the hetero-Diels−Alder reaction[14,15] and the carbonyl transformation into oxime ethers.[16,17]

Another reaction that is recently emerging as an attractive "click" process is the addition of thiols to alkenes, which is called thiol−ene coupling or thiol−ene click reaction.[18] Thiol−ene chemistry has many of the attributes of alkyne−azide click chemistry, such as tolerance to many different reaction conditions, facile synthetic strategies and clearly defined reaction pathways. Therefore, the thiol−ene click reaction has been utilized for a range of applications, including cross-linked polymeric matrices, such as hydrogels,[19,20] polymer and nanoparticle functionalization,[21,22] dendrimer synthesis,[23] and nanoprinting and patterning.[24,25]

While both the alkyne−azide and thiol−ene click chemistries have been extensively used for a large number of applications, ranging from optical components[26] and biomaterials,[27,28] these chemistries have not been thoroughly investigated for the controlled assembly of supramolecular structures in the form of 3-D degradable nanoparticles. Traditional mechanisms for forming polyester materials, such as solvent displacement[29] and salting-out[30] methods do not allow for the controlled implementation of network architectures and, therefore, the tailoring of release profiles and degradation of such materials has been challenging, which is an indirect result of the self-assembly of the linear polyester. We found that the formation of degradable nanoparticles via intermolecular chain cross-linking reactions show a tremendous versatility regarding nanoscopic size control, degradation and linear release kinetics of therapeutics.[22,31] These factors will be become increasingly critical to facilitate delivery systems to respond to the demands of different types of cancers in single and combination therapy, as we have recently investigated in preliminary tumor growth delay studies,[32] and for the optimum treatment for a broad range of diseases requiring targeted and nontargeted sustained delivery systems. As the importance of this methodology for nanoparticle formation is recognized, we sought to investigate other chemistries that are capable to facilitate the nanoparticle formation with the same efficiency as the reported epoxide-amine cross-linking reaction.[33] Similar to the epoxide amine reaction, reactions were selected that do not require the addition of any other reagents, are considered high yielding and are carried out under mild reaction conditions, such as alkyne−azide and thiol−ene "click" chemistries.

This work reports the successful systematic preparation of degradable nanoparticles in a variety of distinct nanoscopic size dimensions using the traditional alkyne−azide click chemistry and the more recently developed thiol−ene click reaction by covalently cross-linking alkyne or allyl functionalized linear polyesters with bisazides or dithiols, respectively. We demonstrate that the intermolecular chain cross-linking reaction for nanoparticle formation is driven by the efficiency of the cross-linking reaction and procedures such as the "click" reactions are highly adaptable,

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novel processes for this technique, providing yet another source to these valuable biomaterials.

Characterization. ^{1}H NMR spectra were obtained from a Bruker AC300 Fourier Transform Spectrometer, with $CDCl_3$/TMS as the solvent. ^{13}C NMR spectra were obtained from a Bruker AC400 Fourier Transform Spectrometer with $CDCl_3$ as the solvent. Gel-permeation chromatography (GPC) was carried out with a Waters chromatograph system equipped with a Waters 2414 refractive index detector, a Waters 2481 dual λ absorbance detector, a Waters 1525 binary HPLC pump, and four 5 mm Waters columns (300 mm × 7.7 mm), connected in series with increasing pore size (100, 1000, 100 000, and 1 000 000 Å respectively). All runs were performed with tetrahydrofuran (THF) as the eluent at a flow rate of 1 mL/min. For dynamic light scattering (DLS), a Malvern Nano ZS system by Malvern Instruments (Malvern Zetasizer Nanoseries, Malvern, U.K.) was employed at a fixed angle of 90° at 25 °C, taking the average of three measurements. The particles were diluted with toluene to a concentration, which gave the desired number of counts in order to obtain a good signal-to-noise ratio. Samples for transmission electron microscopy (TEM) imaging were prepared by dissolving 0.5 mg of nanoparticles in 1 mL of 2-propanol and 0.4 mL of acetonitrile. The samples were sonicated for 5 min and were stained with 5 drops of 3% phosphotungstic acid. The carbon grids were prepared by slowly dipping an Ultrathin Carbon Type-A 400 Mesh Copper Grid (Ted Pella, Inc., Redding, CA) into the particle solutions three times and drying the grid at ambient temperature. A Philips CM20T transmission electron microscope operating at 200 kV in bright-field mode was used to obtain TEM micrographs of the polymeric nanoparticles.

Materials. Reagent chemicals were purchased from Aldrich (St. Louis, MO), and Acros (Morris Plains, NJ) and used as received, unless otherwise stated. Spectra/Por Dialysis membrane and SnakeSkin Pleated Dialysis Tubing, regenerated cellulose, were purchased from Spectrum Laboratories Inc. and Pierce Biotechnology, respectively. Analytical TLC was performed on commercial Merck plates coated with silica gel 60 F_{254}. Silica gel for column chromatography was Sorbent Technologies 60 Å (40−63 μm, technical grade). Monomers α-allyl-δ-valerolactone and α-propargyl-δ-valerolactone were synthesized as reported in the literature.[33]

Synthesis of Copolymer Poly(vl-pvl) (AC). A 25 mL 3-necked round-bottom flask, equipped with stir bar, was sealed with two septa and a gas inlet. The flask was evacuated and refilled with argon three times. Stock solutions of 1.7 M ethanol (EtOH) in THF and 3.7×10^{-2} M tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) in THF were made in sealed Ar(g) purged flasks. Solutions of EtOH (0.13 mL, 0.22 mmol) and $Sn(Oct)_2$ (0.12 mL, 4.3×10^{-3} mmol) were combined in the Ar(g) purged 3-necked round-bottom flask. After stirring the mixture for 20 min, α-propargyl-δ-valerolactone (pvl, 0.35 g, 2.5 mmol) and δ-valerolactone (vl, 1.1 g, 10.0 mmol) were added. The reaction vessel stirred at 105 °C for 48 h. Residual monomer and catalyst were removed by precipitating the polymer into cold diethyl ether to give a golden brown polymer (1.18 g, 81.4%). M_w = 3000 Da, PDI = 1.18. ^{1}H NMR (300 MHz, $CDCl_3$/Me_4Si): δ 4.10 (m,−CH_2−O−), 3.64 (m, CH_3CH_2O−), 2.59 (m, pvl, HC≡CCH_2CH−), 2.35 (m, vl, −CH_2CH_2C(O)O−, pvl, HC≡CCH_2CH−, HC≡CCH_2CH−), 2.03 (m, HC≡C−), 1.68 (m, pvl and vl, −CHCH_2CH_2-), 1.25 (t, CH_3CH_2O−); ^{13}C NMR (400 MHz, $CDCl_3$): δ 174.7 (avl, −C(O)−), 172.7 (pvl, −C-(O)−), 134.6 (H_2C=CH−), 116.4 (H_2C=CH−), 82.0, 70.7, 68.9, 38.7, 35.9, 27.5, 23.9, 23.6, 21.3, 20.9.

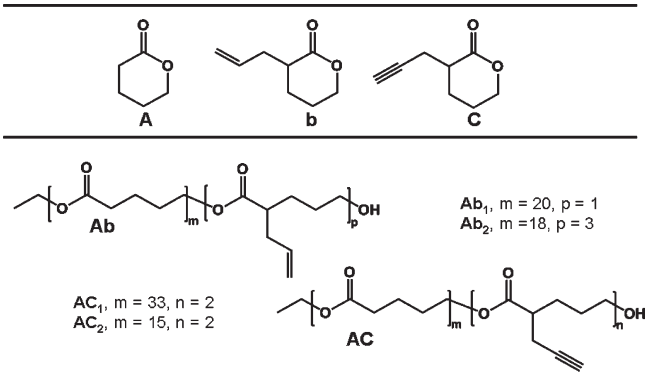
Nanoparticle Formation Using Alkyne−Azide "Click" Cross-Linking with 1,8-Diazide-3,5-dioxaoctane. Poly(vl-pvl), **AC**, (43.5 mg, M_w = 3000 Da, PDI = 1.18) was added to a vial, which was then sealed and purged with argon. To the vial were added 1,8-diazide-3,5-dioxaoctane (10.4 mg, 5.2×10^{-5} mmol) dissolved in anhydrous dimethylformamide (0.8 mL) and copper(I) bromide (52 μL, 7.0×10^{-2} M solution in DMF) were added. The reaction mixture stirred for 24 h at room temperature. Residual azide and copper bromide were removed by dialyzing with SnakeSkin Pleated Dialysis Tubing (MWCO = 25,000) against 50/50 dichloromethane/methanol to yield particles (43.4 mg). DLS: D_H = 87.5 ± 5.3 nm. ^{1}H NMR (300 MHz, $CDCl_3$/Me_4Si): The significant change is the reduction of the alkyne proton at 2.03 ppm and the appearance of signals at 3.83, 3.66, and 3.40 ppm corresponding to the protons of the PEG linker and the signal at 7.49 ppm due to the protons from triazole formation as a result of cross-linking. All other aspects of the spectrum are similar to that of **AC**.

Synthesis of Copolymer Poly(valerolactone−allylvalerolactone) (Poly(vl-avl)), Ab. A 50 mL 3-necked round-bottom flask, equipped with stir bar, was sealed with two septa and a gas inlet. The flask was evacuated and refilled with $Ar_{(g)}$ three times. Stock solutions of 1.7 M ethanol (EtOH) in THF and 3.7×10^{-2} M tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) in THF were made in sealed Ar(g) purged flasks. Solutions of EtOH (0.32 mL, 5.41×10^{-1} mmol) and $Sn(Oct)_2$ (0.30 mL, 1.12×10^{-2} mmol) were combined in the nitrogen purged 50 mL flask. After the mixture was stirred for 30 min, α-allyl-δ-valerolactone (1.16 g, 8.32 mmol) and δ-valerolactone (vl, 2.50 g, 24.97 mmol) were added. The reaction vessel was stirred at 105 °C for 48 h. Residual monomer and catalyst were removed by dialyzing with Spectra/Por dialysis membrane (MWCO = 1000) against CH_2Cl_2 to give a golden brown polymer, **Ab** (3.24 g, 88%). M_w = 3400 Da, PDI = 1.16. ^{1}H NMR (300 MHz, $CDCl_3$/Me_4Si): δ 5.7 (m, H_2C=CH−), 5.09 (m, H_2C=CH−), 4.09 (m, −CH_2−O−), 3.65 (m, CH_3CH_2O−), 2.35 (m, vl −CH_2CH_2C(O)O−, avl H_2C = $CHCH_2CH$−, H_2C=CHCH_2CH−), 1.68 (m, avl and vl −CHCH_2CH_2−), 1.25 (t, CH_3CH_2O−). ^{13}C NMR (400 MHz, $CDCl_3$): δ 174.6 (avl −C(O)−), 172.7 (vl -C(O)−), 134.6 (H_2C=CH−), 116.4 (H_2C=CH−), 63.3, 44.3, 35.9, 33.1, 27.5, 25.9, 23.6, 20.9.

Nanoparticle Formation Using Thiol−Ene Cross-Linking with 3,6-Dioxa-1,8-octanedithiol. A solution of poly(vl-avl), **Ab**, (0.14 g, M_w = 3042 Da, PDI = 1.18) dissolved in CH_2Cl_2 (0.2 mL) was added to a solution of 3,6-dioxa-1,8-octanedithiol (19.6 μL, 0.12 mmol) in CH_2Cl_2 (24.4 mL). The reaction mixture was heated for 12 h at 44 °C. Residual dithiol was removed by dialyzing with SnakeSkin Pleated Dialysis Tubing (MWCO = 10,000) against CH_2Cl_2 to yield particles (0.13 g). DLS: D_H = 72.6 ± 2.8 nm. ^{1}H NMR (300 MHz, $CDCl_3$/Me_4Si): The significant change is the reduction of the allyl protons at 5.06 and 5.77 ppm and the appearance of signals at 3.65 and 2.69 ppm corresponding to the protons neighboring the thiols of the PEG linker after cross-linking. All other aspects of the spectrum are similar to that of **Ab**.

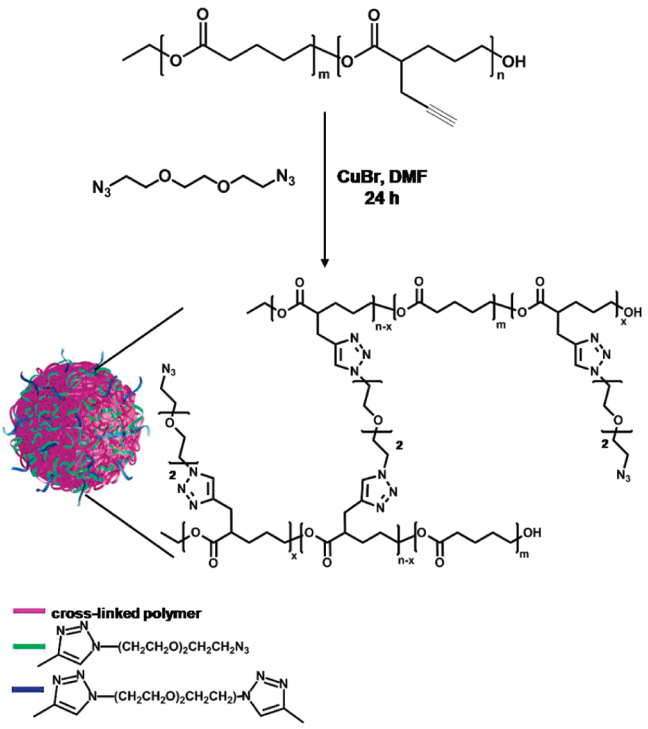
General procedure for *in vitro* cytotoxicity of nanoparticles (MTT assay). The cytotoxicity of the nanoparticles was evaluated using an MTT assay. HeLa cells were cultured in Eagle's Minimum Essential Medium supplemented with 10% heat inactivated fetal bovine serum, L-glutamine, penicillin streptomycin sulfate antibiotic-antimycotic mixture and gentamicin. Cells were maintained at 37 °C with 5% CO_2 in a 95% humidity incubator. The cells were seeded in a 96-well plate in 100 μL of media per well at a density of 10 000 cells/well and incubated for 24 h. The media was then replaced with 100 μL of phenol red free medium-containing nanoparticles at different concentrations in triplicate and

Table 1. Multifunctional Linear Polyester Precursors Ab and AC



incubated for 24 h. After incubation, the nanoparticle containing media was removed, the cells were rinsed three times with DPBS, to avoid interference in the assays, and 100 μL of fresh phenol red free media was added, followed by 10 μL of MTT solution (5 mg/mL). The cells were incubated for 4 h, after which time the medium was carefully removed. To the resulting purple crystals, 100 μL of DMSO was added to lyse the cells, and the reaction was incubated for 10 min at 37 °C. The MTT absorbance was measured at 540 nm using a Synergy HT Multimode microplate reader (Bio Tek Instruments, Winooski, VT). Optical densities measured for wells containing cells that received no nanoparticle were considered to represent 100% viability. Results are expressed as the mean ± SD of viable cells.

Scheme 1. Nanoparticle Formation Using Alkyne−Azide Click Cross-Linking



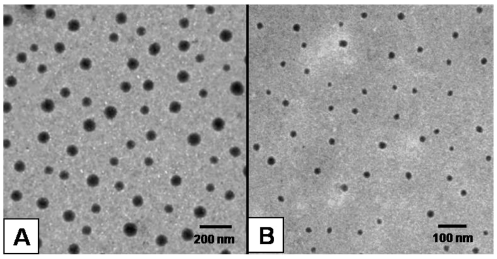
Discussion

Particle formation using the traditional alkyne−azide click chemistry starts with the synthesis of a low molecular weight linear polyester with pendant alkyne groups, poly(valerolactone-propargylvalerolactone) (poly(vl-pvl)) and was synthesized by copolymerizing α-propargyl-δ-valerolactone with commercially available δ-valerolactone through ring-opening polymerization (ROP) with ethanol and tin(II) 2-ethylhexanoate, as the initiator and catalyst respectively, to result in a linear polymer with 5% alkyne functionality, **AC$_1$** (Table 1).

Upon copolymerization, the alkyne functionalized polymer precursors were cross-linked with a bisazide, 1,8-diazide-3,6-dioxaoctane to test the ability to form and control nanoparticle formation. A series of experiments were completed in which **AC$_1$**, dissolved in dimethylformamide (DMF), was reacted with copper(I) bromide and either 2, 4, or 8 equiv of azide per pendant alkyne in the polymer in a one-pot reaction (Scheme 1), equivalent to the previously published one-pot intermolecular cross-linking technique.[22] The reactions were carried out at 45 °C for 24 h and subsequently dialyzed to remove unreacted starting materials. As evidenced by dynamic light scattering (DLS), each of the experiments was successful in forming well-defined monodisperse nanoparticles (Figure 1). With 2 equiv of azide per alkyne group, a particle of 40 ± 4 nm was obtained, whereas 4 equiv of azide per alkyne moiety results in a 88 ± 5 nm particle. In addition to DLS, the spherical morphology of the particles was observed by transmission electron microscopy (TEM), as shown in Figure 1. Both DLS and TEM underline the versatility of the alkyne−azide click approach to prepare well-defined nanoparticles in narrow nanoscopic size dimensions, controlled by the equivalents of bisazide. Analogous to the cross-linking reaction using the epoxide-amine chemistry the nanoparticles are completely soluble in organic solvents and inherit the solubility of the linear polymer precursor, an advantage for particle characterization and modification. With further characterization of the particles via ^{1}H NMR, we also confirmed nanoparticle formation, as evidenced by a reduction of the signal at 2.03 ppm due to the alkyne proton and the appearance of the peak at 7.49 ppm due to the proton from triazole formation as a result of cross-linking (Figure 2). ^{1}H NMR was also able to indicate an increase in particle size, since the signal at 3.40, 3.66, and 3.83 ppm characteristic of the methylene protons of the bisazide linker and the peak at 7.49 ppm corresponding to the triazole protons intensified as the particles became larger in size with the consecutive increase of the azide cross-linker and is in agreement with the intermolecular cross-linking process demonstrated for the epoxide- amine cross-linking reaction as previously reported.[33] The ability to control the size of the nanoparticles was examined in further detail by performing a second set of experiments in which we investigated the effect of increasing the amount of alkyne incorporated in the linear polymer from 5% to 12%. For these reactions, the equivalencies of the bisazide were varied from 2 to 8 azides per alkyne group in the polymer, **AC$_2$**, containing 12% of the alkyne cross-linking unit, and were found to result in well-defined larger particles as compared to those formed from **AC$_1$**. For example, 84 nm particles were obtained with 2 azides per alkyne moiety from **AC$_2$**, whereas for **AC$_1$**, 4 azides per alkyne group needed to be used in order to achieve 88 nm particles.

We can conclude that with the alkyne−azide cross-linking chemistry nanoparticle size can be both controlled by the equivalencies of the azide cross-linker and the percentage of the incorporated alkyne entities in the linear polymer, in the same fashion as it was observed in the epoxide-amine cross-linking reaction. With this, we have found another suitable reaction to perform the intermolecular cross-linking reaction in a controlled manner.

In addition to its simplicity and efficiency, the alkyne−azide cycloaddition has been shown to proceed with ease under mild conditions, such as room temperature.[34] Therefore, we further explored the capability of preparing particles at ambient temperature for the possible future encapsulation of sensitive bioactive cargo, such as peptides and proteins, during particle formation. To achieve this goal, a series of reactions were completed at room



Azide/ 1 Alkyne	Diameter (nm) AC_1 particles 45 °C	Diameter (nm) AC_2 particles 45 °C	Diameter (nm) AC_1 particles room temp.	Diameter (nm) AC_2 particles room temp.
2	40 ± 4	84 ± 6	21 ± 2	46 ± 4
4	88 ± 5	178 ± 17	37.1 ± 2.9	76 ± 7
8	183 ± 14	367 ± 27	**78 ± 5**	179 ± 13

Figure 1. (Top) TEM images of $\mathbf{AC_1}$ and $\mathbf{AC_2}$ particles (**A**) $\mathbf{AC_1}$ particles prepared at 45 °C with 4 equiv azide and (**B**) $\mathbf{AC_2}$ particles prepared at room temperature with 2 equiv azide. (Bottom) Nanoparticle sizes of $\mathbf{AC_1}$ and $\mathbf{AC_2}$ particles formed at 45 °C or room temperature.

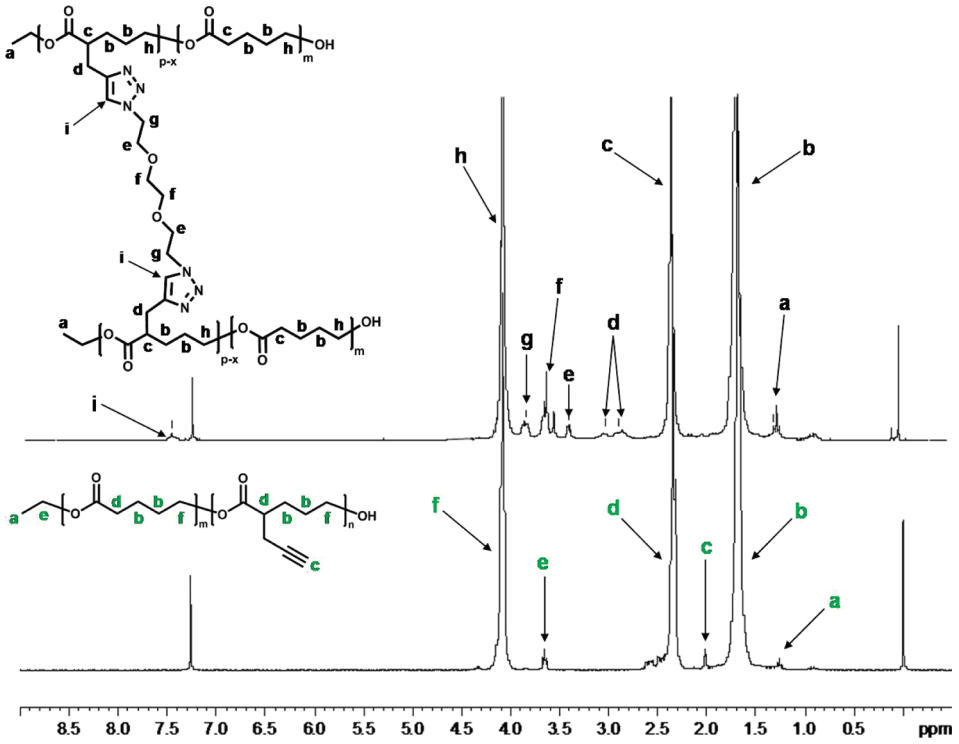


Figure 2. (Top) ^{1}H NMR spectra of $\mathbf{AC_1}$ particles, 88 nm and linear $\mathbf{AC_1}$ linear polymer precursor (Bottom).

temperature in which $\mathbf{AC_1}$ was coupled for 24 h in DMF with either 2, 4, or 8 equiv of azide per alkyne in the presence of copper bromide. Analysis by DLS demonstrated that the reduction in temperature was efficient in producing well-defined slightly smaller particles (Figure 1) as compared to those prepared at 45 °C.

By increasing the percent of incorporated alkyne groups in the linear polymer, the size of the particles can be systematically increased as was seen with the case of polymer $\mathbf{AC_2}$. Using 2 equiv of azide per alkyne group, 45 nm particles can be prepared, whereas 4 equiv of azide per alkyne unit were required to obtain 37 nm particles (Figure 1). Although the ambient reaction temperature resulted in marginally smaller particles, it is evident that there remains excellent control over the formation of well-defined particles with varied distinct nanoscopic size dimensions.

The copper-catalyzed click reaction between azides and alkynes is ideal for many applications, however it has been reported that the copper(I) has the undesirable side effect of being cytotoxic if not removed thoroughly from the product.[19,35] For the removal of the catalyst, we needed to dialyze the nanoparticles for several days until the solution became color free. To evaluate the cellular cytotoxicity of the purified nanoparticles, the cell viability was evaluated by utilizing an MTT assay (Figure 5). The cellular toxicity was determined by incubating HeLa cells for 24 h with varying concentrations of particles in triplicate ranging from 5 mg mL^{-1} to 0.001 mg mL^{-1}. As seen in Figure 5A, the nanoparticles did not cause significant cytotoxicity against the HeLa cell line as compared to other polyester materials reported in the literature.[31] The experimental TC_{50} value for the particles was found to be approximately 0.88 mg mL^{-1}.

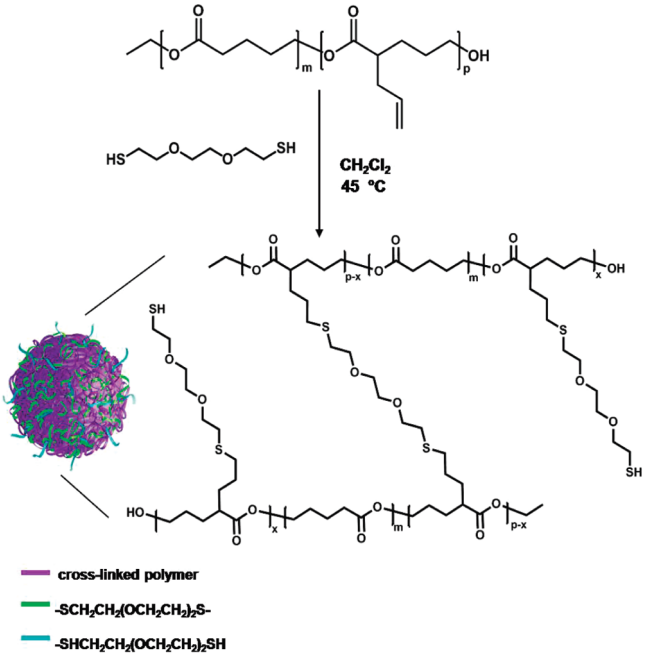
In addition to the alkyne−azide chemistry, there has been significant development with click reactions that do not require any metal catalyst while exhibiting all of the beneficial properties of the copper-catalyzed alkyne−azide click reaction, such as the thiol−ene click reaction. Therefore, we were drawn to use

the thiol−ene reaction for the formation of nanoparticles since this chemistry can be utilized with no catalysts or other toxic reagents and is known to be highly efficient.[36,37]

Assembly of the nanoparticles using thiol−ene click coupling begins in a very similar manner to that of the particles formed by the alkyne−azide reaction, with the synthesis of a low molecular weight linear copolymer, however, with pendant allyl groups instead of alkyne units. Integration of the allyl moieties, the critical functionality for cross-linking, was accomplished by copolymerizing α-allyl-δ-valerolactone (b) with δ-valerolactone (A) via ROP as previously reported[33] to afford poly(valerolactone-allylvalerolactone) (poly(vl-avl)), $\mathbf{Ab_1}$, with 5% allyl groups incorporated (Table 1).

Subsequent to copolymerization, the allyl functionalized linear polyester was primed for covalently cross-linking with dithiol in order to form nanoparticles (Scheme 2). In comparison to our previously published cross-linking technique, the allyl functionalized linear polymer, poly(valerolactone−allylvalerolactone) (poly(vl-avl)), still required oxidation to provide the epoxide functionality for cross-linking with diamine (2,2′-(ethylenedioxy)-bis(ethylamine)). However, with our current approach, we have eliminated this oxidation reaction and simplified particle formation.

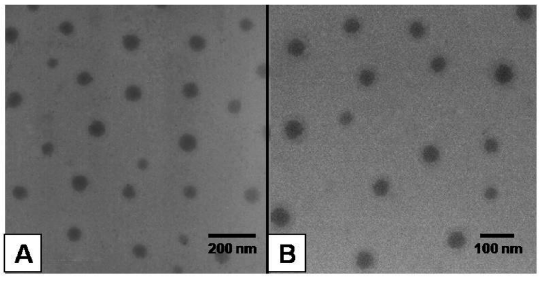
Scheme 2. Nanoparticle Formation Using Thiol−Ene Click Cross-Linking



To form the nanoparticles, the linear polymer **Ab** was dissolved in CH_2Cl_2, added to a solution of 3,6-dioxa-1,8-octanedithiol in CH_2Cl_2 and then heated at 45 °C for 24 h (Scheme 2), a straightforward one-pot technique for forming well-defined particles.[22] The cross-linker 3,6-dioxa-1,8-octanedithiol, the dithiol version of 1,8-diazide-3,6-dioxaoctane used in the aforementioned approach, was specifically chosen to compare the particles prepared from the two click cross-linking processes. In addition, we have previously shown that the thiol−ene click chemistry, in the absence of catalyst or initiator, proceeded with the greatest efficiency with slightly elevated temperatures[22] and the particle formation was carried out at 45 °C.

As a starting point for achieving monodisperse particles with controlled nanoscopic size dimensions, we first investigated the effect of varying the amount of dithiol available for cross-linking with the linear polymer. Equivalents of dithiol cross-linker were increased from 1 to 8 thiols per allyl group in $\mathbf{Ab_1}$. As a result of varying the equivalents of thiol, the size of the particle can be precisely controlled as seen by DLS analysis (Figure 3). By relating the size of the particle to the equivalents of dithiol used, it is apparent that there is a polynomial increase in nanoscopic diameter. The morphology of the particles was investigated by transmission electron microscopy (TEM) and shown in Figure 3. From both DLS and TEM, we could conclude that this thiol−ene cross-linking method affords particles with well-defined size and shape.

Since the nanoparticles inherit their linear polyester precursors' solubility, the particles were also characterized by ^{1}H NMR. The incorporation of the dithiol was confirmed by the significant reduction of the alkene protons of the allyl group at 5.0 and 5.7 ppm and the appearance of a peak at 2.69 ppm, predominantly due to the methylene protons adjacent to the sulfide functionalities. In addition, the methylene protons neighboring the oxygens in the linker appeared at 3.64 ppm. Both of these resonances



Thiol/ 1 Allyl	Diameter (nm) Ab_1 particles 12 h	Diameter (nm) Ab_1 particles 24 h	Diameter (nm) Ab_2 particles 12 h	Diameter (nm) Ab_2 particles 24 h
1	21 ± 2	29 ± 2	57 ± 2	186 ± 9
2	50 ± 3	68 ± 3	109 ± 9	257 ± 14
3	73 ± 3	89 ± 4	143 ± 11	301 ± 16
4	109 ± 7	123 ± 8	189 ± 16	350 ± 21
6	253 ± 11	300 ± 13	344 ± 23	501 ± 35
8	397 ± 18	429 ± 20	692 ± 47	884 ± 78

Figure 3. (Top) TEM images of $\mathbf{AC_1}$ and $\mathbf{AC_2}$ particles (**A**) $\mathbf{AC_1}$ particles prepared from a 12 h reaction with 3 equiv thiol and (**B**) $\mathbf{AC_2}$ particles formed from a 12 reaction with 1 equiv of thiol. (Bottom) Nanoparticle sizes of $\mathbf{AC_1}$ and $\mathbf{AC_2}$ particles obtained from 12 or 24 h reactions at 45 °C.

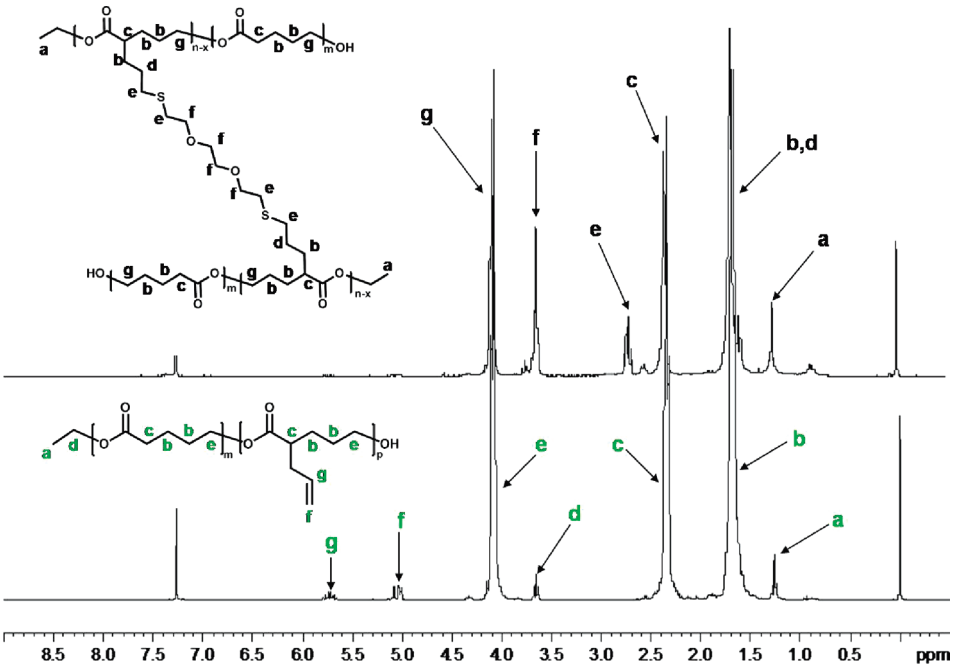


Figure 4. (Top) ^{1}H NMR spectra of $\mathbf{AC_1}$ particles, 123 nm (24 h) and linear $\mathbf{AC_1}$ linear polymer precursor (Bottom).

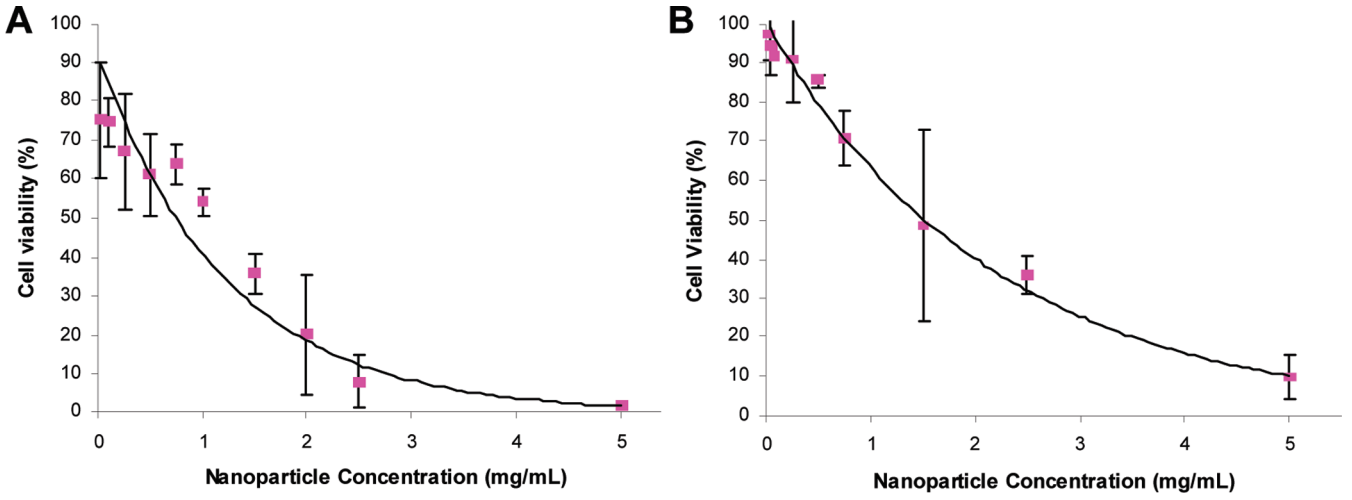


Figure 5. Cytotoxicity of particles on HeLa cells using the MTT assay. Fitted curves show HeLa cell viability incubated for 24 h with (A) alkyne−azide cross-linked particles and (B) thiol−ene cross-linked particles.

along with the reduction in the allyl protons verify successful cross-linking of the dithiol with the linear polymer.

In conjunction with examining the effect of dithiol equivalents for nanoparticle formation, we also studied the result of altering the quantity of allyl groups incorporated in the linear polymer precursor. For this investigation, another polymer $\mathbf{Ab_2}$ with an increased amount of 12% allyl groups was synthesized. With this linear polymer, a series of experiments were completed in which the equivalents of thiol were increased from 1 to 8. In accordance with the alkyne−azide cross-linking approach, the nanoparticle size dimension systematically increased with the higher percentage of allyl groups in the linear polymer precursors and with the increase in equivalents of thiol, as was seen by DLS (Figure 3).

The biocompatibility of the particles formed from thiol−ene cross-linking was evaluated utilizing an MTT assay (Figure 5B). Cytotoxicity against HeLa cells was determined as a function of nanoparticle concentration (ranging from 0.001 mg mL^{-1} to 5 mg mL^{-1}), and untreated cells were used as the negative control. After 24 h exposure to the particles, cell viability was measured and the particle concentration causing a 50% cytotoxic effect was found to be approximately 1.5 mg mL^{-1}. In contrast with the alkyne−azide cross-linked particles, which had a TC_{50} of 0.88 mg mL^{-1}, the thiol−ene cross-linked particles exhibited a lower cytotoxicity which could be due to the absence of residual copper catalyst. The cytotoxicity of the previously reported particles from epoxide−amine cross-linking reactions had a TC_{50} of 1.00 mg mL^{-1},[31] which is between the toxicity of the particles produced with "click" chemistry approaches.

Comparing the particle formation at 45 °C using the thiol−ene and alkyne−azide click chemistries, it is evident that the thiol−ene coupling results in reasonably larger particles than the alkyne−azide reaction. For example, for thiol−ene cross-linking, 4 equiv of thiol per allyl group in $\mathbf{Ab_1}$ results in 123 nm particles, whereas for the alkyne−azide reaction, 88 nm particles were obtained with 4 equiv of azide per alkyne unit in $\mathbf{AC_1}$. As a further assessment of the thiol−ene cross-linking efficiency, a series of reactions were carried out at 45 °C for only 12 h, which remains a sufficient amount of time to form discrete particles, with equivalences

of cross-linker varied from 1 to 8 thiols per allyl group. While reducing the reaction time from 24 to 12 h decreased the sizes of the particles, the sizes were, however, still slightly larger than those prepared by the alkyne−azide coupling with similar equivalencies of cross-linker (Figure 1 and Figure 3). From these results, we can conclude that the thiol−ene click reaction, which did not require the use of a catalyst or initiator, was very effective in forming well-defined nanoparticles as compared to the alkyne−azide click cross-linking.

In summary, this work has demonstrated that "click" chemistries, such as the alkyne−azide reaction and the thiol−ene coupling, are effective cross-linking reactions to form well-defined polyester particles via intermolecular chain collapse in selected nanoscopic size dimensions. Linear polyester precursors were effectively transformed into particles of selected size dimensions by reacting the polymers' pendant alkyne groups with 1,8-diazide-3,6-dioxaoctane in the presence of copper(I) bromide and varying both the equivalencies of azide and the amount of alkyne groups incorporated in the linear polymer. The fact that the alkyne−azide cross-linking reaction can be successfully carried out at room temperature will be an important asset for the further development of this technique to encapsulate sensitive cargo. In comparison to the alkyne−azide chemistry, the thiol−ene "click" reaction, performed without the use of any catalyst or initiator, was equally efficient in the performance of a controlled intermolecular cross-linking reaction. For particle formation, the pendant allyl moieties of linear poly(vl-avl) were coupled with 3,6-dioxa-1,8-octanedithiol to give distinct spherical nanoparticles in controlled size dimensions. Once more, it was found that particle size can be effectively directed by the quantity of incorporated allyl units in the polymer and the equivalencies of thiol cross-linking partner. Additionally, by reducing the duration of the thiol−ene cross-linking reaction from 24 to 12 h, we have demonstrated the ability to efficiently form discrete nanoparticles in half the amount of time in contrast to the alkyne−azide cross-linking reaction at 45 °C. Both the alkyne−azide and thiol−ene click chemistries have led to the development of two novel intermolecular chain cross-linking reactions for the successful preparation of well-defined spherical particles, which is further evidence for the synthetic utility of click chemistry in materials science.

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