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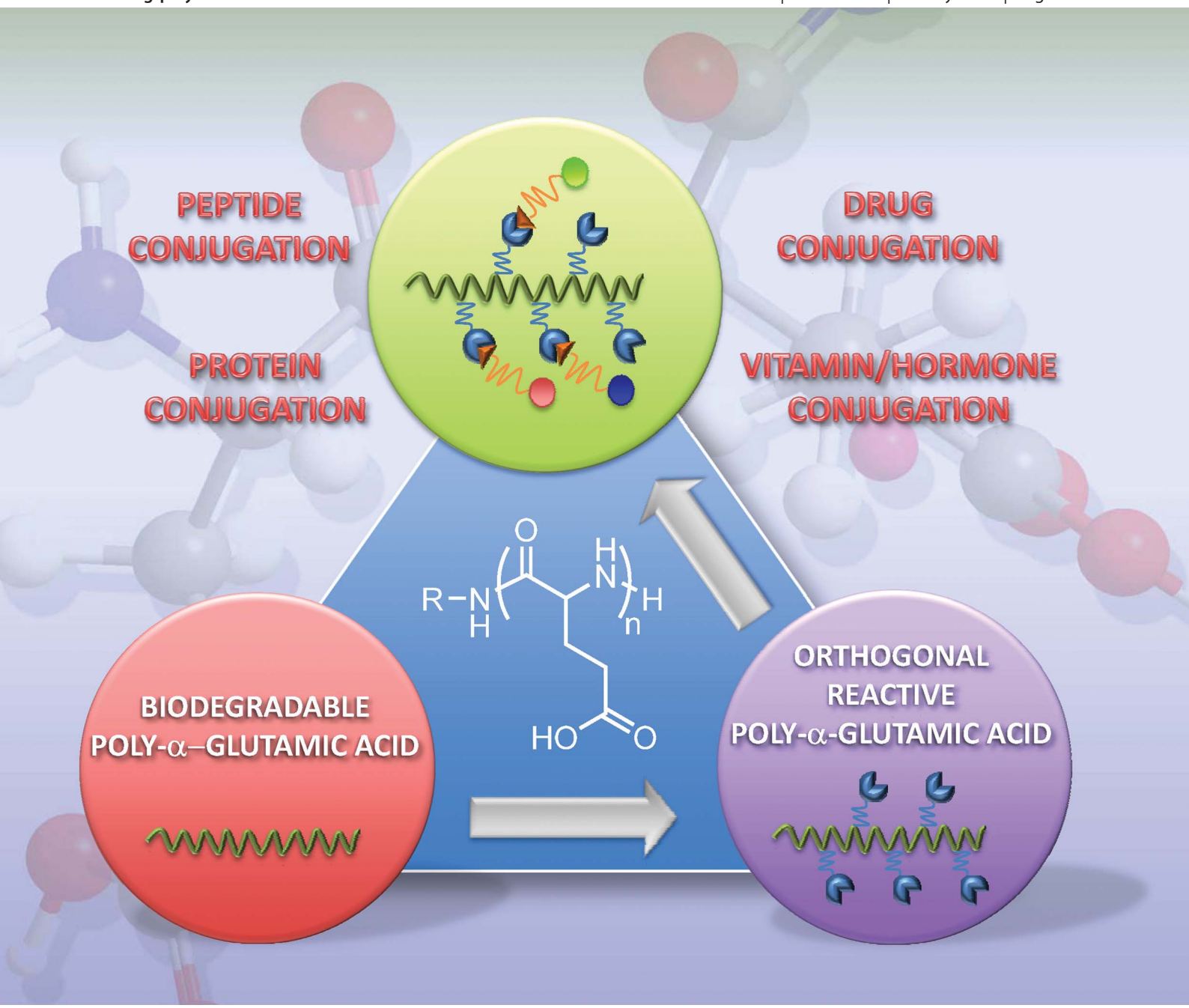

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PAPER

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A versatile post-polymerization modification method for polyglutamic acid: synthesis of orthogonal reactive polyglutamates and their use in "click chemistry"

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In this article we describe a versatile methodology for the synthesis of polyglutamic acid (PGA) derivatives bearing orthogonal reactive sites. The reactive groups enable selective conjugation chemistry by copper catalyzed azide–alkyne coupling (CuAAC). PGA was derived in aqueous media as well as in organic media using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMTTM) salts. The spectra of attached chemical moieties ranges from simple PEGylation with 2,5,8,11,14,17,20-heptaoxadocosan-22-amine (mEG(6)NH_2) to the incorporation of propargylamine, 11-azido-3,6,9-trioxaundecan-1-amine (NH_2-EG(2)N_3), and 20-azido-3,6,9,12,15,18-hexaoxaicosan-1-amine (NH_2-EG(6)N_3). Herein, it is demonstrated that the degree of functionalization can be easily controlled within this one pot reaction. Additionally, we report conditions for the CuAAC with various PGA derivatives, which can be employed for site-specific conjugation of either hydrophilic or hydrophobic compounds.

Introduction

Advances in polymer chemistry and nanoscience over the last decade provide a new basis for the development of innovative delivery and imaging techniques with significant potential to bring benefits to patients and open new markets for pharmaceutical industry. Polymer therapeutics are amongst the most successful nanomedicines.[1] Polymeric drugs,[2] polymer–protein conjugates[3] and polymer–aptamer conjugates[4] are currently in routine clinical use with a demonstrated clinical benefit. Also, polymer–drug conjugates are in advanced clinical trials.[5] Most polymer conjugates use *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers, PEG or more recently polyglutamic acid (PGA) as carriers.[2,6] Biopersistent carriers (PEG, HPMA) present disadvantages if chronic parenteral administration and/or high doses are required as they have potential to generate 'lysosomal storage disease' syndrome. Preclinical evidence of intracellular vacuolation[6] and complement activation[7] including certain PEG–protein conjugates raises awareness about the potential advantage of biodegradable polymers regarding safety benefit apart from the possibility to use higher molecular weight (M_w) carriers allowing PK optimization.[8]

The most prominent examples of biodegradable polymers in use are polypeptides, hyaluronic acid, polyesters, dextrins, polyacetals and hydroxyethyl starch.[8,9] Beside the choice of an appropriate polymer the chemistry for the attachment of bioactive molecules also has to be considered. In this respect, conjugation chemistry of polymers to natural or non-natural agents is of major importance. The incorporation of reactive sites becomes even more demanding whenever orthogonal reactive groups are mandatory for site-specific conjugation of bioactive compounds (peptides, proteins).

Conjugation strategies can be divided into two categories: chemical[10] and biological ligation.[11] Among the chemical strategies, the CuAAC method has been widely applied[12–15] since it is a versatile but easy to perform methodology for site-specific chemical ligation of complex molecules to polymers.

There are two approaches to introduce reactive groups into polymers for CuAAC; the (co)polymerization of reactive monomers[16] and the post-polymerization modification techniques.[17] While copolymerization may offer a certain control about distribution of reactive groups among the polymer chain, the post-polymerization modification is usually much easier to perform, since the synthesis of monomer bearing the reactive group can be avoided.

In this work we would like to introduce a versatile post-polymerization modification methodology allowing the introduction of alkyne, azide or PEG moieties into PGA. PGA has already demonstrated its potential as a hydrophilic polymer in drug delivery. A PGA paclitaxel conjugate is currently in Phase III clinical trials (Opaxio™, Cell Therapeutics Inc.) and has been

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recently designated by FDA as an 'orphan drug' in combination with radiotherapy for the treatment of glioblastoma.[5]

The conjugation chemistry used here includes the attachment of alkyne, azide or PEG and is based on the *in situ* activation of carboxyl functionalities of PGA by 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium (DMTMM) chloride in aqueous solution.[18] Furthermore, optimized conditions for the CuAAC of hydrophilic as well as hydrophobic molecules to PGA derivatives are reported.

Experimental section

All chemicals were of reagent grade, obtained from Aldrich and used without further purification, unless indicated otherwise. Polyglutamic acid (PGA) ($M_n = 17\,900$, $M_w/M_n = 1.13$) was synthesized according to reported methodology;[19] 2,5,8,11,14,17,20-heptaoxadocosan-22-amine (mEG(6)NH_2), 11-azido-3,6,9-trioxaundecan-1-amine (NH_2-EG(2)N_3), and 20-azido-3,6,9,12,15,18-hexaoxaicosan-1-amine (NH_2-EG(6)N_3) were obtained from Iris Biotech Gmb. All solvents were of analytical grade. Dimethylformamide (DMF) was purified prior to use.

Deuterated chloroform-d_1, DMSO-d_6, DMF-d_7 and D_2O were purchased from Deutero GmbH.

Preparative SEC was performed using Sephadex G-25 superfine from GE as well as PD MiniTrap G-10™ columns containing 2.1 mL of Sephadex™ G-10.

The polymers were characterized by size exclusion chromatography (SEC). For SEC measurements in DMF containing 1 g L^{-1} of lithium bromide as an additive, an Agilent 1100 series system was used with a flow rate of 1 mL min^{-1} at 30 °C as an integrated instrument, including three HEMA-based columns ($10^5/10^3/10^2$ Å porosity) from MZ-Analysentechnik GmbH, a UV (275 nm) and an RI detector. Calibration was achieved with well defined poly(methyl methacrylate) (PMMA)/DMF standards, provided by Polymer Standards Service (PSS)/Mainz Germany.

^{1}H-NMR spectra were obtained at 300 MHz using a FT-spectrometer from Bruker and analyzed using the MestreNova 6.2 software.

The click reactions were monitored by IR using the Nicolet™ 380 FT-IR spectrometer from Thermo Scientific.

Method for post-polymerization modification using DMTMM chloride

Synthesis of PGA-*co*-propargyl polyglutamic acid amides. In a one neck round bottom flask fitted with a stir bar and a stopper, 200 mg of PGA (1.55 mol of glutamic acid repeat unit, 1 eq.) were suspended in 10 mL of MilliQ water. Afterwards 128.7 mg (0.465 mmol, 0.3 eq.) of DMTMM chloride was added and dissolved in 5 mL of MilliQ water. After 10 minutes, 51 mg (0.93 mmol, 0.6 eq.) of propargylamine were added and the pH was adjusted to 8 by adding some drops of 1 M $NaHCO_3$ solution. The reaction was allowed to proceed overnight, stirring at room temperature. After that, as all byproducts are soluble in acid aqueous solution, the copolymer was purified by acid–base precipitation, resuspended in MilliQ water and lyophilized yielding a colorless amorphous solid. Yield: 80–90%.

^{1}H-NMR (300 MHz, D_2O, 11% mod) δ: 4.30–4.02 (m, 1H), 3.81 (s, 0.26H)*, 2.48 (s, 0.09H)*, 2.35–2.02 (m, 2H), 2.01–1.65 (m, 2H) (corresponding with Fig. 1 and Table 1, entry 3).

Synthesis of PGA-*co*-mEG(6) polyglutamic acid amides. The synthesis was done according to PGA-*co*-propargyl polyglutamic acid amide. In a one neck round bottom flask fitted with a stir bar and a stopper, 200 mg of PGA (1.55 mol of glutamic acid repeat unit, 1 eq.) were suspended in 10 mL of MilliQ water. Afterwards 128.7 mg (0.465 mmol, 0.3 eq.) of DMTMM chloride was added and dissolved in 5 mL of MilliQ water. After 10 minutes, 315 mg (0.93 mmol, 0.6 eq.) of mEG(6) were added and the pH was adjusted to 8 by adding some drops of 1 M $NaHCO_3$ solution. The reaction was allowed to proceed overnight, stirring at room temperature. After this, as all byproducts are water soluble, either ultrafiltration (with a membrane of 3.000 M_w) or size exclusion chromatography with Sephadex G25 columns was done in order to purify the copolymer.

A colorless amorphous solid was yielded after freeze-drying the sample. Yield: 80–90%.

^{1}H-NMR (300 MHz, D_2O, 8% mod) δ: 4.33–4.19 (m, 1H), 3.95–3.78 (m, 2.08H), 3.77–3.49 (m, 0.16H), 3.34 (s, 0.24H), 2.41–1.76 (m, 4H).

Synthesis of PGA-*co*-EG(2/6)N_3 polyglutamic acid amides. The synthesis was done according to PGA-*co*-propargyl polyglutamic acid amide. In a one neck round bottom flask fitted with a stir bar and a stopper, 200 mg of PGA (1.55 mol of glutamic acid repeat unit, 1 eq.) were suspended in 10 mL of MilliQ water. Afterwards 128.7 mg (0.465 mmol, 0.3 eq.) of DMTMM chloride was added and dissolved in 5 mL of MilliQ water. After 10 minutes 0.93 mmol, 0.6 eq. of EG(2/6)N_3 were added and the pH was adjusted to 8 by adding some drops of 1 M $NaHCO_3$ solution. The reaction was allowed to proceed overnight, stirring at room temperature. After this, as all byproducts are water soluble, either ultrafiltration (with a membrane of 3.000 M_w) or size exclusion chromatography with Sephadex G25 columns was done in order to purify the copolymer.

A colorless amorphous solid was yielded after freeze-drying the sample. Yield: 80–90%.

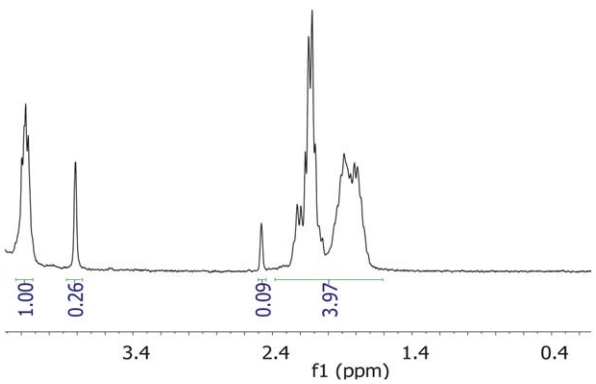


Fig. 1 ^{1}H-NMR spectra of PGA-*co*-propargyl polyglutamic acid amide.

Table 1 Characteristics of the polymers synthesized

PGA-*co*-propargyl PGA amide	Alkyne content (calc.)	Alkyne content (^{1}H-NMR)	M_n	M_w	PDI
1	50%	31%	19 487	22 020	1.13
2	30%	18%	18 818	21 264	1.13
3	20%	11%	18 458	20 858	1.13
4	10%	6%	18 201	20 567	1.13
PGA-*co*-EG(2/6)N_3 PGA amide	Azide content (calc.)	Azide content (^{1}H-NMR)	M_n	M_w	PDI
5 ($n = 2$)	60%	30%	26 225	29 634	1.13
6 ($n = 2$)	20%	15%	22 336	25 240	1.13
7 ($n = 2$)	15%	11%	20 948	23 671	1.13
8 ($n = 6$)	50%	34%	33 569	37 932	1.13
9 ($n = 6$)	30%	18%	26 191	29 596	1.13
10 ($n = 6$)	20%	16%	25 269	28 554	1.13
PGA-*co*-mEG(6) PGA amide	OligoEG content (calc.)	OligoEG content (by ^{1}H-NMR)	M_n	M_w	PDI
11	200%	87%	56 675	64 043	1.13
12	140%	73%	50 434	56 991	1.13
13	100%	49%	39 736	44 901	1.13
14	60%	30%	31 266	35 330	1.13
15	20%	8%	21 459	24 248	1.13

^{1}H-NMR (300 MHz, D_2O, 15% mod EG(2)N_3) δ: 4.28–4.07 (m, 1H), 3.65–3.51 (m, 1.50H), 3.48 (t, $J = 5.6$ Hz, 0.30H), 3.40–3.30 (m, 0.30H), 3.25 (d, $J = 4.9$ Hz, 0.30), 2.29–2.00 (m, 2H), 1.98–1.65 (m, 2H) (corresponding to Fig. 2 and Table 1, entry 6).

Methodology for copper catalyzed alkyne–azide coupling (CuAAC) of PGA derivatives

(a) CuAAC conditions for the coupling of hydrophobic peptides/drugs. In a two neck round bottom flask fitted with a stirrer bar and a stopper, 1 eq. of copolymer (PGA and EG(2)N_3, EG(6)N_3 or propargylamine in each case) sodium salt was dissolved in MilliQ water. After that, the corresponding amount for the desired % of substitution of the clickable agent was added in dry DMF solution. Then, five equivalents of sodium ascorbate in MilliQ water solution were added ($M_w = 198.11$ g mol^{-1}). Then the mixture was degassed by performing two freeze–pump–thaw cycles. One equivalent of $CuSO_4$ ($M_w = 249.68$ g mol^{-1}) was weighed under N_2 flow and added in MilliQ H_2O solution to the reaction mixture. The final complete mixture, containing a DMF–H_2O proportion of 4 : 1, was degassed by performing another freeze–pump–thaw cycle and left to react at 40 °C in an oil bath protected from light.

(b) CuAAC conditions for the coupling of water-soluble molecules. The synthesis was done according to (a) but using previously degassed MilliQ water.

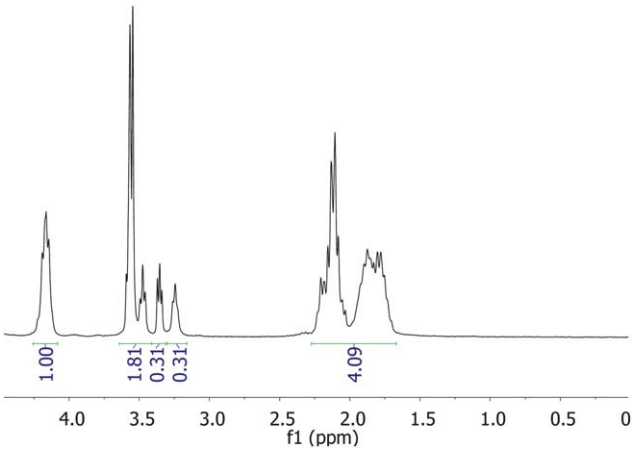


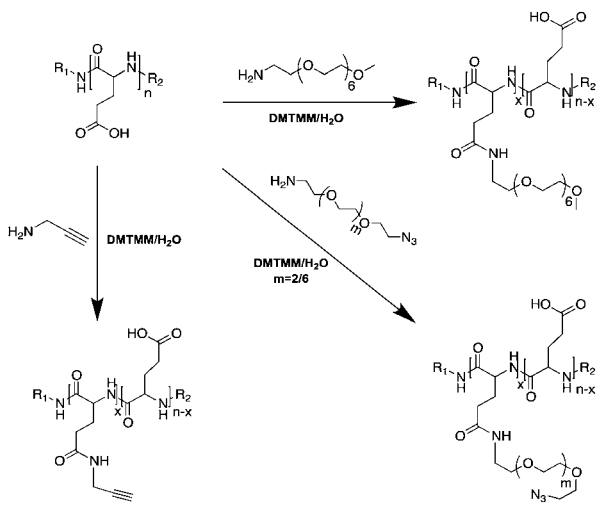
Fig. 2 ^{1}H-NMR spectra of PGA-*co*-EG(2)N_3 polyglutamic acid amide.

^{1}H-NMR (300 MHz, D_2O) δ: 7.79 (s)*, 4.30–4.02 (m, 1H), 3.79 (s)*, 3.63–3.46 (m)* 2.46 (s)*, 2.35–2.02 (m, 2H), 2.01–1.65 (m, 2H) when $NH_2PEG(n)N_3$ was coupled to PGA-*co*-propargyl polyglutamic acid amide.

*The integration of peaks corresponds to the amount of functionalization (PEG, alkyne or azide). When PGA-*co*-EG(2/6)N_3 polyglutamic acid amide polymers were used, the propargyl singlet at 2.46 ppm disappeared. The peak at 7.79 ppm corresponds to the proton of the triazole ring formed in the CuAAC, and was used to determine coupling efficiencies.

Results and discussion

Since the synthesis of controlled biodegradable polymer conjugates for biomedical applications is desired, the post-polymerization modification of PGA with various amines using DMTMM chloride was performed in aqueous media. Therefore, modifications on the procedure reported by Michielsen and Thompson[20] have been performed to adjust



Scheme 1 Synthesis of reactive PGA derivatives and their use for site-specific conjugation.

the protocol for PGA. The activation of the carboxylic acids within the polymer backbone was carried out by using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMTMM chloride). Afterwards, the selected amine was added into the reaction mixture leading to the corresponding modified polymer (see Table 1). The reaction is described in Scheme 1.

The mechanism of action of DMTMM chloride consists of the formation of the activated ester with the release of 4-methylmorpholine in the first step. An amide bond is then formed between the activated ester and the amine present. Once conversion was fully achieved (usually 16 h), different procedures for the purification of the resulting conjugates were explored. One of them was based on acid–base precipitation, PGA is insoluble as carboxylic acid but soluble as sodium salt. Since all by-products were water soluble, the resulting polymers could be easily purified by precipitation in acidic water (pH $\sim$ 3–4) and re-dissolved with sodium bicarbonate. The process was repeated three times and after freeze-drying pure colorless PGA sodium salt could be collected.

Additionally, ultrafiltration by using a membrane of 3.000 M_w cut off was also explored since the polymers with a higher content of mEG(6) (>30%) cannot be precipitated under the above described conditions. Likewise precipitation, ultrafiltration yielded pure colorless PGA derivatives after freeze-drying. Finally, size exclusion chromatography (SEC) using Sephadex G25 columns was also investigated as an alternative purification method. In all three cases, the product was obtained pure and in comparable yields (>80%), but, it could be said that ultrafiltration was preferable at a large scale whereas Sephadex G25/PD10 columns were the preferred methodology at a small scale.

Once purified the percentage of modification achieved was determined (see Table 1).

In PGA-*co*-propargyl polyglutamic acid amide, the alkyne content was quantified by integration of the signals from propargyl alkyne (3.81 ppm CH_2– and 2.48 ppm acetylenic proton) in relation to the signals of PGA (2.40–1.52 ppm PGA side chain and 4.01 ppm α-proton) encountered in the ^{1}H-NMR spectra in deuterated water (see Fig. 1). In the case of PGA-*co*-mEG(6) polyglutamic acid amide, and PGA-*co*-EG(2/6) N_3 polyglutamic acid amides the oligoethyleneglycol content was quantified by integration of the corresponding signals of the ethylenglycol unit in the ^{1}H-NMR spectra in deuterated water, in comparison with the corresponding signals of PGA (see Fig. 2).

The peaks at 3.26, 3.35 and 3.48 ppm correspond to the –CH_2– protons near to the amide group and the azide group. The larger peak at 3.55 ppm belongs to the –CH_2– of the oligoethyleneglycol chain plus one of the triplet corresponding to the terminal CH_2 protons. This latter signal changes in its integration whenever the modification is done with EG_2 (two units of ethyleneglycol in the inside chain which correspond with 10 protons) or EG_6 (which corresponds with 50 protons). Thus, in comparison with the protons of PGA, the % of substitution of each polymer can be easily calculated. The characteristics of all PGA derivatives are displayed in Table 1. Overall, linking efficiencies are comparable or even better than the post-polymerization modification of poly(acrylic acid) PAA as reported by Michielsen and Thompson[20] and are around 50–70%. Only the sterically demanding mEG(6) amine led to a reduced linking efficiency of 45% at high degrees of substitution (87%).

These newly synthesized PGA derivatives have been used to develop a conjugation protocol for the attachment of either hydrophobic or hydrophilic molecules employing the CuAAC methodology. The coupling reaction was monitored by FT-IR by the disappearance of the asymmetric vibration band. Fig. 3 displays a representative FT-IR spectrum. Time zero represents the pure azide in the absence of the corresponding alkyne reagent. As can be seen, the asymmetric vibration band was observed at 2485 cm^{-1}, and this band

Table 2 Reaction conditions used during CuAAC

Experiment			Solv.	T °C	Catalyst ($CuSO_4$–NaA)	Eq.[a]	LE[b]
1	PGA-*co*-propargyl PGA amide	+N_3-EG(2)NH_2	H_2O	25	0.3/0.5	320%	0%
2			H_2O	40	0.3/0.5	320%	88%
3			H_2O	40	1/5	640%	97%
4			DMF–H_2O	40	0.3/0.5	640%	48%
5			DMF–H_2O	40	1/5	29%	67%
6		+N_3-EG(6)NH_2	H_2O	40	1/5	640%	96%
7	PGA-*co*-EG(2)N_3 PGA amide	Propargylamine	H_2O	25	0.3/0.5	314%	0%
8			H_2O	40	0.3/0.5	314%	0%
9			DMF–H_2O	60	1/5	418%	33%
10			DMF–H_2O	60	1/10	29%	55%
11	PGA-*co*-EG(6)N_3 PGA amide	Propargylamine	H_2O	25	0.3/0.5	314%	0%
12			H_2O	40	0.3/0.5	314%	0%
13			DMF–H_2O	40	1/5	29%	67%
14		Propargyl acrylate	DMF–H_2O	40	1/5	2.411%	99%

[a] Eq.: equivalents of clickable molecule per polymer. The % corresponds with the % calculated from modified G.A. units (bearing N_3 or alkyne depending on the polymer used) that will react theoretically. [b] L.E: linking efficiency calculated taking into account the previous percentage, and the % achieved.

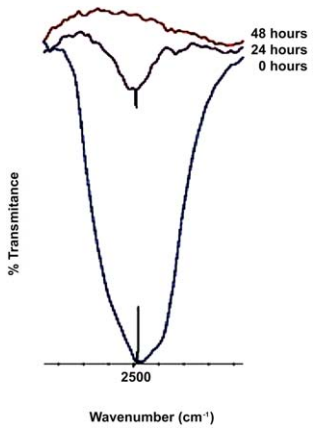


Fig. 3 Representative FT-IR-spectra of a CuAAC reaction between a polymeric azide and a low molecular weight alkyne.

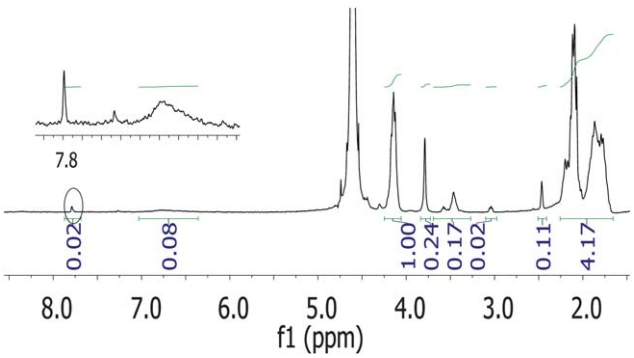


Fig. 4 ^{1}H-NMR spectrum of the CuAAC reaction product between PGA-*co*-propargyl PGA amide and NH_2-EG(2)N_3. The peak surrounded by a circle is associated with the proton of the triazole ring formed during the reaction.

was disappearing with time up to 48 h when it was no longer detectable.

Finally, the polymers were purified according to the above-mentioned methodologies. In all cases colorless polymers could be obtained after freeze-drying from solution.

Reaction conditions have been optimized to nearly quantitative linking efficiencies by screening different solvents, temperatures, catalysts and concentrations (see Table 2).

Besides monitoring the conversion of azides by IR the linking efficiency of the CuAAC reaction was also monitored by ^{1}H-NMR by correlating the integration of triazole proton signal – which is formed during the CuAAC – with the α-proton of PGA (see Fig. 4).

Summarizing, CuAAC reaction on PGA derivatives was achieved in aqueous as well as organic solutions. The use of $CuSO_4$–sodium ascorbate (1/5) as a catalyst at 40 °C was the optimal condition for CuAAC in aqueous media yielding quantitative conversions. The use of DMF–H_2O mixtures (4 : 1) with $CuSO_4$–sodium ascorbate (1/5) also allowed conjugation of hydrophobic but DMF soluble peptides/drugs. The linking efficiency was always lower when DMF–H_2O mixtures were used (55–67%), but acceptable, reproducible and predictable. This effect was attributed to intra-chain aggregation during the conjugation of hydrophobic compounds making it increasingly difficult to reach the conjugation site due to steric hindrance.

Conclusions

In this article we have reported a versatile methodology for the synthesis of polyglutamic acid (PGA) derivatives bearing orthogonal reactive sites, *e.g.* azides and alkynes in combination of the carboxyl functionalities of PGA. Additionally, we have described the linkage of water as well as DMF soluble compounds to PGA, which could be used for site-specific conjugation of a variety of bioactive agents of different nature (*i.e.* peptides, proteins, drugs). Therefore, the described protocols enable a versatile and controlled approach towards the development of next generation PGA-based polymer therapeutics.

Acknowledgements

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