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(71) Applicant: SYNGENTA CROP PROTECTION AG
[CH/CH]; Rosentalstrasse 67, 4058 Basel (CH).(72) Inventors: **PERRIER, Sebastien, Paul, Edmond**; Gibbet Hill Road, Coventry CV4 7AL (GB). **WHITFIELD, Richard, Bradley**; Vladimir-Prelog-Weg 1-5/10, 8093 Zürich (CH). **COOK, Alexander, Beattie**; Gibbet Hill Road, Coventry CV4 7AL (GB). **OMEDES PUJOL, Marta**; Syngenta, Jealotts Hill International Research Centre, Bracknell Berkshire RG42 6EY (GB). **LOCZENSKI ROSE, Vanessa**; Jealott's Hill International Research Centre, Bracknell Berkshire RG42 6EY (GB). **BURNS, James, Adam**; Sir William Lyons Road, Coventry CV4 7EZ (GB). **HADDLETON, David, M**; University of Warwick Department of Chemistry, Coventry Gibbet Hill CV4 7AL (GB).

(74) Agent: SYNGENTA INTERNATIONAL AG; Schwarzwaldallee 215, B4.08.152, 4058 Basel (CH).

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(57) **Abstract:** The present invention relates to a composition comprising a (hydrolysable) polymer plus a single dsRNA sequence or a mixture of dsRNA sequences; and to the use of such a composition for controlling pest growth and/or plant infestation. More particularly, the present invention relates to polymers capable of forming a complex with polynucleotides, particularly double-stranded RNA (dsRNA). The polymers may increase the stability of dsRNA, which increases its efficacy in RNA interference (RNAi) mediated gene silencing, particularly in plant pest organisms.

FORMULATIONS

The present invention relates to a composition comprising a (hydrolysable) polymer plus a single dsRNA sequence or a mixture of dsRNA sequences; and to the use of such a composition for controlling pest growth and/or plant infestation. More particularly, the present invention relates to polymers capable of forming a complex with polynucleotides, particularly double-stranded RNA (dsRNA). The polymers may increase the stability of dsRNA, which increases its efficacy in RNA interference (RNAi) mediated gene silencing, particularly in plant pest organisms.

WO 2016/201523 details the release of dsRNA at basic pH, moreover, the release occurs by the neutralisation of positively charged cations within a clay based compound, however, unlike the present invention, the monomers used in the complex are non-hydrolysable. US 2015/0024488 A1 describes the protection of nucleic acids from enzymatic degradation by complexation to an amphiphilic triblock copolymer. The complex may also be used for gene silencing and efficient transfection of a nucleic acid, however, the release cannot be controlled by hydrolysis of the polymeric structure and also requires a hydrophilic block within the complex. G Wenyi *et al* in Biomacromolecules Vol. 14, 2013, pp3386-3389, show complexation solely *via* DMAEA, which has been shown to have insufficient hydrolytic and enzymatic stability for stabilisation of dsRNA in soil.

RNAi is a well-established technique used to down-regulate gene expression by using dsRNA or small interfering RNA (siRNA) to trigger degradation of mRNA of a particular gene of interest, thus preventing translation. RNAi has not only provided a means of functionally analysing genes, but has been used for the control of pests. The effectiveness of RNAi for plant pest control is limited by the instability and short half-life of dsRNA in the environment, in particular after application to soil or the locus of a plant. Providing a means of increasing the stability of dsRNA provides a more effective means of controlling plant pest organisms by RNAi mediated down-regulation of gene expression.

Thanks to advances in polymer and materials chemistry, stimuli responsive gene delivery systems have made significant progress in the last few years. These systems are able to respond to various stimuli and either trigger release of the transported nucleic acid, or promote endosomal escape of the carrier to the cell cytoplasm. Examples of endogenous stimuli include: intracellular changes in pH environment, temperature difference and the presence of enzymatic triggers; while exogenous stimuli include: light, ultrasound and even magnetism.

In the area of polymer chemistry, polyamines have recently attracted considerable interest as a consequence of the presence of cationic nitrogen atoms that allow for pH tuning and the formation of pH responsive nanoparticle structures that self-assemble in aqueous solution. These properties render polyamines as good candidates for a wide range of applications such as waste water treatment, paper

making and cosmetics. A major shortcoming of these stimuli-responsive systems lies in the toxicity of the cationic polymers remaining after oligonucleotide release. In response, increasing attention is being directed towards developing degradable or charge altering polymers with biocompatible by-products. Degradability can either be introduced in the polymer backbone, or through degradable polymer side chains. In the early 2000s, the poly(β -amino esters) were prepared *via* Michael addition step growth polymerisation of diamines and diacrylates, resulting in cationic polymers with degradable backbones and cationic groups for DNA complexation. In another example, encapsulated genetic material in poly(lactic-co-glycolic acid) (PLGA) was used to deliver siRNA efficiently through cervical mucus barrier in mice models. Backbone degradable polymers based on oligo(carbonate-*b*- α -amino ester)s were also shown to be efficient gene delivery vectors. Introduction of polymer degradability through the polymer side chains, leading to biocompatible and non-toxic degradation products, has also been studied extensively. The degradable side chain route however also has the added advantage of being able to incorporate a change in functionality and/or charge with side chain degradation. For example, polymers synthesised from 2-(dimethylamino)ethyl acrylate (DMAEA) have been investigated for the complexation and release of nucleic acids *via* hydrolysis of the ester connection between acrylate backbone and side chains. Poly(DMAEA) proved to be an attractive polymer for non-viral gene delivery whose initial structure combines hydrolysable side chains with cationic groups. Upon hydrolysis, cationic side chains turn into negatively charged acrylic acid moieties which enhance nucleic acid release *via* electrostatic repulsion. The self-catalysed hydrolysis of pDMAEA in water was initially reported to reach a limiting degree of hydrolysis of 60 to 70% after one week in aqueous conditions at room temperature. More recent studies confirmed that the hydrolysis and nucleic acid release occurs rapidly (1 to 10 hours) and is consistent with a self-catalysed mechanism at a rate that is independent of pH, salt concentration or any other external stimulus. A number of strategies have been employed to lengthen this release time, but it is yet to be extended past 72 hours.

The advantageous opportunities observed by the application of linear polymers with a hydrolysable unit, have been shown to be beneficial when applied to star polymers. Star polymers are of particular interest both in academic and industrial fields as a consequence of their potential applications as viscosity modifiers, catalyst supports, polymer therapeutics, drug carriers and additives. In

comparison with their linear counterparts, star polymers possess additional unique properties thanks to their compact structures and high arm density. The major challenge in the synthesis of well-defined star polymers is bimolecular termination due to star-star coupling. Cu(0)-mediated RDRP has already been employed to yield well-defined stars, including the synthesis of stars homopolymers in a biphasic system, where star-star coupling has been significantly suppressed. However, to date non-functional monomers have been employed (typically butyl acrylate) in all cases, thus limiting the

applications of the resultant materials. This patent describes star polymers which include functional monomers, thus broadening the methodology.

Branched polymers are a special class of polymer architecture characterised by their high branching densities and one-pot synthetic methodologies. The branched polymer topology imparts a number of favourable properties compared to their linear polymer equivalents including: high surface functionality, globular conformation, low intrinsic viscosities, high solubilities and interesting rheological modifying properties. This has led to branched polymers being increasingly important for industrial applications over the past 20–30 years.

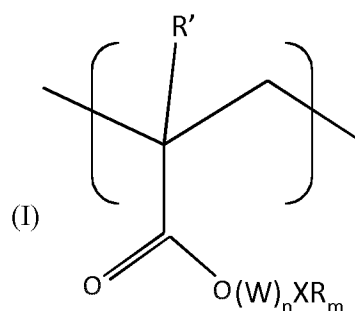
One of the most well-known examples of a hyperbranched polymer formed from ABn polycondensation is the commercial polymer Boltron, synthesised from the monomer bis(methylol)propionic acid (bis-MPA). Boltron hyperbranched polymers with multiple surface hydroxyl functional groups have been synthesised, which have been used in a large number of applications, both as the native polymer and also post-polymerisation modified *via* the hydroxyl groups to impart further functionality or different solubility properties. In this case control over the reaction (resulting molecular weight, molecular weight distribution, and degree of branching) can be achieved by addition of monomer in discrete portions, later developed into the ‘slow monomer addition’ method.

Synthesis of branched polymers *via* a double monomer methodology, $A_n + B_m$, can lead to gelation at high conversions, and critical concentrations. These syntheses also require careful optimisation of the ratio of functional groups, monomer concentrations, purity of reagents, reaction time and temperature, in order to achieve controlled and reproducible reactions of high molecular weights without purification methods. One potential benefit of this synthetic strategy however, is the range of much more readily available monomers.

Radical chain growth polymerisation methods have always produced branching in some cases through the radical polymerisation side reactions of intramolecular backbiting, intermolecular transfer to polymer, and polymerisation of vinyl terminated disproportionation products. However, introduction of branching in radical polymerisations through design was more recently established. Polymerisation of a vinyl monomer bearing an initiating group allows polymerisation through the vinyl group and also through the initiating site, leading to the formation of highly branched polymers. This self-condensing vinyl polymerisation is termed SCVP and has been extended to various chain growth polymerisation methods, such as nitroxide mediated polymerisation (NMP), reversible addition chain transfer polymerisation (RAFT), atom transfer radical polymerisation (ATRP) and ring opening polymerisation (ROP).

The present invention provides novel hydrolysable polymers suitable for stabilising and subsequently releasing dsRNA in a biologically active form.

Accordingly, the present invention provides a composition comprising a polymer comprising (preferably consisting of; i.e. no other monomer units) monomer units of formula (I); and a single dsRNA sequence or a mixture of dsRNA sequences (preferably the polymer is complexed to the single dsRNA sequence or the mixture of dsRNA sequences):



where

$\text{R}' = \text{H}$ or CH_3 ; W is CH_2 or $\text{CH}_2\text{CH}_2\text{O}$; when W is CH_2 then n is an integer from 2 to 5; when W is $\text{CH}_2\text{CH}_2\text{O}$ then n is an integer from 1 to 100 (suitably from 1 to 20; more suitably from 1 to 8; most suitably it is 1 or 2); X is N , N^+ , P or P^+ ; m is 2 when X is N or P ; m is 3 when X is N^+ or P^+ ; and

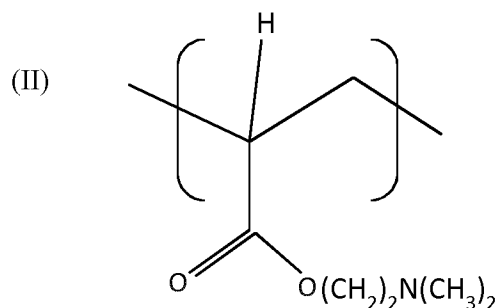
each R is independently a linear or branched C_{1-6} alkyl group or, when X is N , then XR_m may be a saturated ring comprising up to six carbon atoms and up to a further two heteroatoms each independently selected from O and N (suitably XR_m is morpholinyl, pyrrolidinyl or piperidinyl);

provided that R' is CH_3 when W is CH_2 , n is 2, X is N and R is CH_3 (that is R' is not H when W is CH_2 , n is 2, X is N and R is CH_3).

Preferably the polymer has an average M_n greater than 2kDa (more suitably greater than 5kDa; even more suitably greater than 10kDa).

Preferably each R is independently a linear or branched C_{1-6} alkyl (more preferably C_{2-4} alkyl) group; preferably, each R is the same.

The present invention also provides a composition comprising a polymer comprising (preferably consisting of; i.e. no other monomer units) monomer units of formula (I) as defined above and monomer units of formula (II); and a single dsRNA sequence or a mixture of dsRNA sequences (preferably the polymer is complexed to the single dsRNA sequence or the mixture of dsRNA sequences):



The number average molecular weight (M_n) of a polymer sample is defined as the total weight of all the polymer molecules in the sample divided by the total number of polymer molecules in the sample.

The weight average molecular weight (M_w) of a polymer sample denotes the sum of the products of the molar mass of each fraction (i.e. each polymer molecule type) multiplied by its weight fraction.

- 10 The dispersity ($\mathcal{D}$) is the molecular weight distribution of a sample, which is defined by the ratio of the weight-average molecular weight of the sample to the number-average molecular weight of the sample.

The polymers of the present invention will preferably have a number average molecular weight greater than 1×10^3 g/mol (more suitably greater than 1×10^4 g/mol).

- 15 Preferably the polymer has an average M_n greater than 2kDa (more suitably greater than 5kDa; even more suitably greater than 10kDa).

Suitably the monomer units are copolymerised randomly or as blocks.

The polymer may have a linear, star, dendrimer, branched or hyperbranched architecture.

- 20 Suitably the numerical ratio of units of formula (I) to units of formula (II) is from 1:99 to 99:1; preferably from 5:95 to 95:5; more preferably from 10:90 to 90:10; and even more preferably from 20:80 to 80:20.

The present invention also provides a method of substantially retaining or otherwise preserving the biological activity of a dsRNA present in a degradative environment, comprising complexing a single dsRNA or mixture of dsRNA sequences and a polymer as defined above.

- 25 Suitably the polymer controls the rate of hydrolysis and thus release of dsRNA.

Suitably, the complexation of dsRNA to the positively charged polymer can show enhanced protection of the dsRNA in soil. Controlling the ratio of the hydrolytically unstable monomer within the random copolymer enables tuneable release of dsRNA in soil. Similarly, varying the structure of the hydrolysable monomer has also been shown to enable tuneable release of the dsRNA release.

Preferably the dsRNA comprises a strand that is complementary to at least part of a nucleotide sequence of a gene from a plant pest organism. Suitably the dsRNA is from 15 to 1800 (more suitably from 100 to 1200) base pairs in length. Suitably the dsRNA effects post-transcriptional silencing of one or various target genes in a plant pest organism.

- 5 Suitably the dsRNA is produced by synthetic methods or by an organism other than the target organism or pest (bacteria, algae).

A composition of the present invention may be prepared as a suspension concentrate, a wettable powder or a water dispersible granule (for example by spray-drying or granulation).

The composition may be suitable for seed-coating.

- 10 Naturally, the composition may also comprise other conventional formulation components (co-formulants) such as additives, carriers, fillers, dispersants, emulsifiers, adjuvants, solvents etc.

Where the composition is a suspension concentrate, suitably the polymer composition is from 0.2% to 20% (more suitably from 1% to 10%) of the total weight and dsRNA is from 0.1% to 5% (more suitably from 1% to 3%) of the total weight of the composition. The remainder may be selected from
15 cell debris, co-formulants and water.

Where the composition is a wettable powder or a water dispersible granule suitably the polymer composition is from 1% to 80% (more suitably from 10% to 50%) of the total weight and dsRNA is from 0.5% to 20% (more suitably from 5% to 10%) of the total weight of the composition. The remainder may be selected from cell debris and co-formulants.

- 20 A composition according to the present invention may further comprise at least one agronomically acceptable excipient and/or diluent, and optionally at least one pesticidally active ingredient.

The present invention envisages that a composition may be supplied directly to an end user (such as a farmer) as a product already comprising both the dsRNA and the polymer or alternatively the dsRNA and the polymer may be supplied to the end user separately and then mixed together, for example in a
25 spray tank prior to use or application.

The present invention relates to the protection and controlled release of dsRNA *via* tuneable hydrolysis-triggered release. The ratio of hydrolysable to non-hydrolysable monomers can be tuned to determine the rate of dsRNA release in either water or soil.

- In another aspect, the present invention provides a method of controlling a plant pest infestation
30 preferably a subterranean plant pest, comprising:

(a) providing a composition as defined above;

- (b) applying the composition to the locus of a plant, preferably being soil; and
- (c) releasing the dsRNA from the composition in the locus by hydrolysis of the polymer.

Suitably the dsRNA effects post-transcriptional silencing of one or various target genes in said

- 5 subterranean plant pest; preferably the subterranean plant pest is selected from the group consisting of *Diabrotica virgifera virgifera* (Western corn rootworm), *Diabrotica barberi* (Northern corn rootworm), *Diabrotica undecimpunctata howardi* (Southern corn rootworm), *Diabrotica virgifera zea* (Mexican corn rootworm), *Diabrotica speciosa* (cucurbit beetle), nematodes, wireworms, grubs and soil pathogens (such as bacteria and fungi).

- 10 Suitably the subterranean plant pest is a *Diabrotica* insect.

Suitably the dsRNA effects post-transcriptional silencing of one or various target genes in a plant pest organism.

The compositions of the present invention may be used for controlling pest growth and/or plant infestation.

- 15 In another embodiment, the invention relates to a complex of a polymer of the present invention and a polynucleotide, wherein the polymer is capable of being hydrolysed to release said polynucleotide. Such polynucleotides can be DNA or RNA, including but not limited to dsDNA, dsRNA, siRNA, mRNA, and microRNA or any other RNA molecule capable of RNAi gene silencing. Preferably the polynucleotide is a dsRNA.

- 20 In a preferred embodiment, the polynucleotide is a dsRNA, effective in RNAi gene silencing, comprising a strand that is complementary to at least part of a nucleotide sequence of a gene from a plant pest organism.

The phenomenon of RNA interference potentially to silence gene expression is well known.

RNA is relatively unstable and can be rapidly degraded by, for example, ribonucleases which are

- 25 ubiquitously present even outside of cells. A problem with the application of dsRNA either directly to target organisms, or *via* exogenous administration to a locus at which they exist concerns the poor stability of the RNA.

By exogenous application is meant applied to the target organism in such a way that the organism can incorporate it, or that the dsRNA is produced synthetically or in a first organism which is different

- 30 from the target organism and that the target organism incorporates the first organism, or a part thereof comprising the dsRNA so that – either way - the said dsRNA is capable of effecting post-

transcriptional silencing of a gene comprising a nucleotide sequence corresponding to that comprised by the dsRNA.

Exogenous application is distinguished from endogenous production – by which is meant production (generally *via* expression from an appropriate heterologous sequence) in the cells of the target organism of a double stranded RNA capable of post-transcriptionally silencing targeted genes.

Whilst the exogenously applied dsRNA is generally capable of exerting a relevant biological effect within the short term, perhaps even for up to a few days after application, the effect generally rapidly declines with the dsRNA typically having a half-life of only about 12 to 24 hours in soil for example, and further depending on the precise environmental conditions in which it is administered.

Various solutions to this problem have been proposed, including stabilising the dsRNA by encapsulating or otherwise binding it to a polymer which enhances its stability, thus providing for an increased duration of action.

The present invention also provides a composition for preventing plant pest growth and/or infestation comprising the complex of the invention and at least one agronomically acceptable excipient and/or diluent. The composition may further comprise at least one pesticidally active ingredient.

In another embodiment of the invention, there is provided a method for controlling pest growth and/or plant infestation comprising application of a composition of the invention to it or a locus at which it feeds.

There is also provided the method of production of the complex of the invention, comprising mixing wherein the polymer of the invention is mixed with a polynucleotide at a molar ratio between polymer ammonium or phosphonium cationic repeating units and the anionic phosphate groups on dsRNA of (at least 1) to 10; suitably (at least 1) to 2.

Another aspect of the invention provides a method of production of a polymer of the invention by radical polymerisation.

Another aspect of the invention provides a method of production of a polymer of the invention by controlled radical polymerisation.

Subterranean plant pests include those pests that reside in the soil for at least a portion of their life cycle, for example the larval stage.

The dsRNA comprises at least 15 (preferably at least 50) nucleotides that are complementary or at least part of a nucleotide sequence of a target gene in a target organism.

Suitable target genes are those in which post-transcriptional silencing has a detrimental effect on the target organism. For example, altering growth, stunting, increasing mortality, decreasing reproductive capacity or decreasing fecundity, decreasing or causing cessation of feeding behaviour or movement, or decreasing or causing cessation of metamorphosis stage development.

- 5 The target organism may be an insect selected from the group consisting of *Diabrotica virgifera virgifera* (Western corn rootworm), *Diabrotica barberi* (Northern corn rootworm), *Diabrotica undecimpunctata howardi* (Southern corn rootworm), *Diabrotica virgifera zae* (Mexican corn rootworm), *Diabrotica speciosa* (cucurbit beetle), nematodes, wireworms and grubs and appropriate soil pathogens such as bacteria and fungi.
- 10 Another aspect of the invention provides a method of production of a polymer of the invention, by controlled radical polymerisation, including but not limited to nitroxide mediated polymerisation (NMP), reversible addition-fragmentation chain transfer (RAFT), atom transfer radical polymerisation (ATRP) and ring opening polymerisation (ROP), comprising the following steps;
 - i) polymerisation of a reaction mixture comprising; an initiator, at least one monomer, catalyst
 - 15 and solvent;
 - ii) optionally terminating of said polymerisation, optionally by diluting the reaction mixture with solvent, or addition of a polymerisation inhibitor or quenching agent;
 - iii) optionally purifying said polymer by precipitating said polymer in a suitable non-solvent.

- 20 The present invention also provides a method of production of a polymer of the invention, by transition metal mediated reversible deactivation radical polymerisation, comprising the following steps;

- i) polymerisation of a reaction mixture comprising; at least 2 monomers which are not the same, catalyst, solvent and optionally an initiator;
- 25 ii) optionally terminating of said polymerisation, optionally by diluting the reaction mixture with solvent, or addition of a polymerisation inhibitor or quenching agent;
- iii) optionally purifying said polymer by precipitating said polymer in a suitable non-solvent.

wherein step i) is performed at a temperature from about 0°C to about 120°C.

Examples

The invention is further explained by reference to the following non-limiting examples.

30 Example 1. Polymer Synthesis

2-(dimethylamino)ethyl acrylate (DMAEA), 2-(dimethylamino)ethyl methacrylate (DMAEMA), 3-(dimethylamino)propyl acrylate (DMPA), ethyleneglycol dimethacrylate (EGDMA), di(ethylene glycol) diacrylate (DEGDA), 4,4'-Azobis(4-cyanovaleric acid) (ACVA), 1,1'-Azobis(cyclohexanecarbonitrile) (VA088), polyethylenimine branched (bPEI, $M_w \sim 25,000$ by LS, $M_n \sim 10,000$ by SEC), Agarose, Ethidium bromide solution (500 $\mu\text{g/mL}$ in H_2O), were all obtained from Sigma-Aldrich. All other materials were purchased from Fisher Scientific, or Sigma-Aldrich. dsRNA from Syngenta. 2-(((butylthio)-carbonothioyl)thio)propanoic acid (PABTC) was prepared according to a previously reported procedure by Ferguson *et al.*, (C. J. Ferguson, R. J. Hughes, D. Nguyen, B. T. T. Pham, R. G. Gilbert, A. K. Serelis, C. H. Such, B. S. Hawkett, *Macromolecules*, 2005, **38**, 2191-2204). (4-cyano pentanoic acid)yl ethyl trithiocarbonate (CPAETC) was prepared according to a previously reported procedure by Larnaudie *et al.*, (S. C. Larnaudie, J. C. Brendel, K. A. Jolliffe, S. Perrier, *J. Polym. Sci., Part A: Polym. Chem.*, 2016, **54**, 1003-1011). 50X Tris-Acetate-EDTA (TAE) buffer for gel electrophoresis made up at concentration of 2.0M Tris acetate (Sigma Aldrich) and 0.05M EDTA (Sigma Aldrich) in deionised water, pH 8.2 - 8.4, stored at room temperature. Agarose loading buffer for samples (colourless) made up at 30% (vol/vol) glycerol (Sigma Aldrich) in deionised water, stored at room temperature. 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT sodium salt), and Phenazine methosulfate (PMS) were obtained from Sigma. Size Exclusion Chromatography (SEC) was performed in CHCl_3 , using an Agilent 390-LC MDS instrument equipped with differential refractive index (DRI), viscometry, dual angle light scattering, and dual wavelength UV detectors. The system was equipped with 2 x PLgel Mixed D columns (300 x 7.5 mm) and a PLgel 5 μm guard column. The eluent was CHCl_3 with 2% TEA (trimethylamine) additive, and samples were run at 1 mL/min at 30 $^\circ\text{C}$. Analyte samples were filtered through a nylon membrane with 0.22 μm pore size before injection. Apparent molar mass values ($M_{n,\text{SEC}}$ and $M_{w,\text{SEC}}$) and dispersity ($\mathcal{D}$) of synthesized polymers were determined by DRI detector and conventional calibration using Agilent SEC software. Poly(styrene) standards (Agilent EasyVials) were used for calibration. The Kuhn-Mark-Houwink-Sakurada parameter $[\eta]$, relating to polymer conformation in solution was determined from the gradient of the double logarithmic plot of intrinsic viscosity as a function of molecular weight, using the SEC viscometry detector and Agilent SEC software. Proton nuclear magnetic resonance spectra (^1H NMR) were recorded on a Bruker Advance 400 or 300 spectrometer (400 MHz or 300 MHz) at 27 $^\circ\text{C}$, with chemical shift values (δ) reported in ppm, and the residual proton signal of the solvent used as internal standard. Proton-decoupled carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded on a Bruker Advance 400 (100 MHz) at 27 $^\circ\text{C}$, with chemical shift values (δ) reported in ppm, and the residual proton signal of the solvent used as internal standard. Fourier transform infrared spectra (FTIR) were recorded on a Bruker Alpha FTIR ATR.

Synthesis of branched acrylates pDMAEA and pDMAPA

For a typical branched acrylate polymerisation, with the conditions [DMAEA]: [DEGDA]: [PABTC]: [ACVA] = 50: 2.5: 1: 0.1, PABTC (33.3 mg, 0.140 mmol), DMAEA (1 g, 6.98 mmol), DEGDA (74.8 mg, 0.35 mmol), ACVA (3.9 mg, 0.0140 mmol) and dioxane (1.19 ml) were added to a vial deoxygenated by bubbling with nitrogen and left to stir in an oil bath at 70 °C. After 24 hours, the solution was removed from the oil bath and the polymer precipitated in hexane (three times), and dried under vacuum. $M_{w,MALLS} = 299,000$ g/mol, $\bar{D} = 14$ (CHCl₃ SEC, MALLS detector). ¹H NMR spectrum (400 MHz, CDCl₃, δ ppm): 4.15 (m, 2H, -C(O)O-CH₂-CH₂-NMe₂), 2.55 (m, 2H, -C(O)O-CH₂-CH₂-NMe₂), 2.27 (m, 6H, -CH₂-NMe₂), 2.11–0.91 (m, 3H, backbone). ¹³C NMR spectrum (100 MHz, CDCl₃, δ ppm): 174.28 (-C(O)O-), 62.33 (-C(O)O-CH₂-CH₂-), 57.52 (-CH₂-N(CH₃)₂), 45.71 (-N(CH₃)₂), 41.28 (backbone tertiary), 25.02 (backbone -CH₂-). FTIR ν cm⁻¹: 2948 (medium, C-H alkane), 2821 and 2768 (medium, N-CH₃ amine), 1728 (strong, C=O ester), 1455 (medium, C-H alkane), 1251 (medium, C-N, amine), 1153 (strong, C-O ester).

Sample	M: XL : CTA ^a	$M_{n,SEC}$ ^b (g/mol)	$M_{w,SEC}$ ^b (g/mol)	$\bar{D}$ ^b	$M_{w,MALLS}$ ^c (g/mol)	a ^d
pDMAEA	50 : 2.5 : 1	19,000	268,000	14	299,000	0.36
pDMAPA	50 : 2.5 : 1	22,000	168,000	7.6	193,000	0.53

^a Ratio of monomer to crosslinker (EGDMA or DEGDA) to CTA. ^b From CHCl₃ SEC, DRI detector, linear PS standard. ^c Molecular weight from light scattering detection on CHCl₃ SEC. ^d a = Kuhn-Mark-Houwink-Sakurada parameter, from CHCl₃ SEC viscometry detector.

Synthesis of branched methacrylate pDMAEMA

For a typical polymerisation, with the conditions [DMAEMA]: [EGDMA]: [CPAETC]: [VA-088] = 50: 0.95: 1: 0.025, CPAETC (33.5 mg, 0.127 mmol), DMAEMA (1 g, 6.36 mmol), EGDMA (24.0 mg, 0.121 mmol), VA-088 (0.777 mg, 0.00318 mmol), and dioxane (0.974 mL) were added to a vial deoxygenated by bubbling with nitrogen and left to stir in an oil bath at 90 °C. After 24 hours, the solution was removed from the oil bath and the polymer precipitated in hexane (x3), and dried under vacuum. $M_{w,MALLS} = 275,000$ g/mol, $\bar{D} = 8.2$ (CHCl₃ SEC, MALLS detector). ¹H NMR spectrum (400 MHz, CDCl₃, δ ppm): 4.07 (m, 2H, -C(O)O-CH₂-CH₂-NMe₂), 2.57 (m, 2H, -C(O)O-CH₂-CH₂-NMe₂), 2.29 (m, 6H, -CH₂-NMe₂), 2.17–0.74 (m, 5H, backbone). ¹³C NMR spectrum (100 MHz, CDCl₃, δ ppm): 177.36 (-C(O)O-), 63.03 (-C(O)O-CH₂-CH₂-), 57.09 (-CH₂-N(CH₃)₂), 45.79 (-

$N(CH_3)_2$, 44.73 (backbone quaternary), 19.01 (backbone $-CH_2-$), 18.78 (backbone $-CH_3$). FTIR ν cm^{-1} : 2944 (medium, C-H alkane), 2820 and 2769 (medium, N- CH_3 amine), 1722 (strong, C=O ester), 1455 (medium, C-H alkane), 1270-1265 (medium, C-N, amine), 1153 (strong, C-O ester).

Sample	M : XL : CTA ^a	$M_{n,SEC}$ (g/mol) ^b	$M_{w,SEC}$ (g/mol) ^b	$\bar{D}$ ^b	$M_{w,MALLS}$ (g/mol) ^c	a ^d
pDMAEMA	50 : 0.95 : 1	27,000	218,000	8.2	275,000	0.41

- 5 ^aRatio of monomer (M) to crosslinker (XL) (EGDMA or DEGDA) to CTA. ^bFrom $CHCl_3$ SEC, DRI detector, linear PS standard. ^cMolecular weight from light scattering detection on $CHCl_3$ SEC. ^d a = Kuhn-Mark-Houwink-Sakurada parameter, from $CHCl_3$ SEC viscometry detector.

Synthesis of branched copolymer p(DMAEMA₄₀-co-DMAEA₁₀)

- 10 For a polymerisation, with the conditions [DMAEMA]: [DMAEA]: [EGDMA]: [CTA]: [ACVA] = 40: 10: 1.5: 1: 0.05, CPAETC (21.06 mg, 0.0801 mmol), DMAEMA (0.503 g, 3.204 mmol), DMAEA (0.115 g, 0.801 mmol), EGDMA (23.76 mg, 0.120 mmol), ACVA (1.12 mg, 0.0040 mmol), and dioxane (0.673 mL) were added to a vial deoxygenated by bubbling with nitrogen and left to stir in an oil bath at 70 °C. After 24 hours, the solution was removed from the oil bath and the polymer
- 15 precipitated in hexane (x3), and dried under vacuum. $M_{w,MALLS}$ = 134,000 g/mol, $\bar{D}$ = 3.1 ($CHCl_3$ SEC, MALLS detector). ¹H NMR spectrum (400 MHz, $CDCl_3$, δ ppm): 4.13 (m, 2H, $-C(O)O-CH_2-$ CH_2-NMe_2), 2.69 (m, 2H, $-C(O)O-CH_2-CH_2-NMe_2$), 2.29 (m, 6H, $-CH_2-NMe_2$), 2.18–0.77 (m, 5H, backbone). ¹³C NMR spectrum (100 MHz, $CDCl_3$, δ ppm): 177.31 ($-C(O)O-$), 63.02 ($-C(O)O-CH_2-$ CH_2-), 57.07 ($-CH_2-N(CH_3)_2$), 45.78 ($-N(CH_3)_2$), 44.70 (backbone tertiary), 20.02 (backbone $-CH_2-$),
- 20 18.02 (backbone $-CH_3$). FTIR ν cm^{-1} : 2944 (medium, C-H alkane), 2820 and 2769 (medium, N- CH_3 amine), 1722 (strong, C=O ester), 1455 (medium, C-H alkane), 1263-1270 (medium, C-N, amine), 1144 (strong, C-O ester).

Sample	M1 : M2 : XL : CTA ^a	$M_{n,SEC}$ (g/mol) ^b	$M_{w,SEC}$ (g/mol) ^b	$\bar{D}$ ^b	$M_{w,MALLS}$ (g/mol) ^c	a ^d
p(DMAEMA _{0.8} - co-DMAEA _{0.2})	40 : 10 : 0.95 : 1	17,600	54,900	3.1	134,000	0.42

- ^a Ratio of monomer 1 (DMAEMA) to monomer 2 (DMAEA) to crosslinker (EGDMA or DEGDA) to CTA. ^b From $CHCl_3$ SEC, DRI detector, linear PS standard. ^c Molecular weight from light scattering

detection on CHCl₃ SEC. ^d *a* = Kuhn-Mark-Houwink-Sakurada parameter, from CHCl₃ SEC viscometry detector.

Synthesis of branched copolymer p(DMAEMA_{10-co}-DMAEA₄₀)

- 5 For a polymerisation, with the conditions [DMAEMA]: [DMAEA]: [DEGDA]: [CTA]: [ACVA] = 10: 40: 1.5: 1: 0.05, CPAETC (21.06 mg, 0.0801 mmol), DMAEMA (0.126 g, 0.801 mmol), DMAEA (0.458 g, 3.204 mmol), DEGDA (25.68 mg, 0.120 mmol), ACVA (1.12 mg, 0.0040 mmol), and dioxane (0.713 mL) were added to a vial deoxygenated by bubbling with nitrogen and left to stir in an oil bath at 70 °C. After 24 hours, the solution was removed from the oil bath and the polymer
- 10 precipitated in diethyl ether (x3), and dried under vacuum. $M_{w,MALLS}$ = 66,600 g/mol, $\bar{D}$ = 1.7 (CHCl₃ SEC, MALLS detector). ¹H NMR spectrum (400 MHz, CDCl₃, δ ppm): 4.17 (m, 2H, -C(O)O-CH₂-CH₂-NMe₂), 2.65 (m, 2H, -C(O)O-CH₂-CH₂-NMe₂), 2.28 (m, 6H, -CH₂-NMe₂), 2.05–0.85 (m, 3H, backbone). ¹³C NMR spectrum (100 MHz, CDCl₃, δ ppm): ¹³C NMR spectrum (100 MHz, CDCl₃, δ ppm): 174.26 (-C(O)O-), 62.35 (-C(O)O-CH₂-CH₂-), 57.53 (-CH₂-N(CH₃)₂), 45.72 (-N(CH₃)₂), 41.07
- 15 (backbone tertiary), 25.02 (backbone -CH₂-). FTIR ν cm⁻¹: 2944 (medium, C-H alkane), 2820 and 2767 (medium, N-CH₃ amine), 1726 (strong, C=O ester), 1455 (medium, C-H alkane), 1263 (medium, C-N, amine), 1155 (strong, C-O ester).

Sample	M1 : M2 : XL : CTA ^a	$M_{n,SEC}$ (g/mol) ^b	$M_{w,SEC}$ (g/mol) ^b	$\bar{D}$ ^b	$M_{w,MALLS}$ (g/mol) ^c	<i>a</i> ^d
p(DMAEMA _{0.2-co} -DMAEA _{0.8})	10 : 40 : 2.5 : 1	18,100	31,100	1.7	66,600	0.53

^a Ratio of monomer 1 (DMAEMA) to monomer 2 (DMAEA) to crosslinker (EGDMA or DEGDA) to CTA. ^b From CHCl₃ SEC, DRI detector, linear PS standard. ^c Molecular weight from light scattering

- 20 detection on CHCl₃ SEC. ^d *a* = Kuhn-Mark-Houwink-Sakurada parameter, from CHCl₃ SEC viscometry detector.

Example 2. Polymer Hydrolysis

- The hydrolysis kinetics of the branched tertiary amine-containing polymers were studied using ¹H
- 25 NMR spectroscopy in D₂O. Polymers (10 mg) were dissolved in D₂O (0.6 mL, pH ~ 7.4), incubated at room temperature, and ¹H NMR spectra were then acquired over a period of 3 weeks (Figure 1). The hydrolysis of DMAEA units in the polymer results in the appearance of sharp peaks at ~3.7 ppm, 2.9 ppm, and 2.6 ppm, due to the creation of dimethylaminoethanol hydrolysis product. Integration of

these peaks in comparison with the peak at 4.2 ppm representing the total sum of monomer units was used to calculate the percentage hydrolysis.

Example 3. RNAi Complexation and Release into Water (Agarose Electrophoresis)

Agarose gel electrophoresis

5 Agarose gels (1% w/v) were prepared with agarose and 1 × Tris-Acetate-EDTA (TAE) buffer with DNase/RNase free water. The solution was cooled on the bench for 5 minutes and 100 µL of 0.5 µg/mL ethidium bromide solution was added. The mixture was poured into the casted agarose tray and a comb inserted. The gel was left to set for a minimum of 30 minutes at room temperature. The agarose gels were run in 1× TAE buffer. The final gel was visualized under UV illumination at 365
10 nm using a UVP benchtop UV transilluminator system. Polyplexes of dsRNA were prepared at various N/P ratios. dsRNA stock solution of 60 µg/mL was prepared in PBS, and polymer stock solution of 300 µg/mL. For polyplex formation: appropriate amount of polymer stock solution and dsRNA stock solution were mixed and made up to a total volume of 100 µL in PBS (final concentration of dsRNA was 0.030 µg/µL, in all solutions). Polyplexes were vortexed and incubated
15 at room temperature for 30 minutes. Prior to loading, 30 µL of loading buffer was added to each sample and 20 µL of polyplexes were loaded into the agarose gel wells. Gel electrophoresis was performed at 100 V for 30 minutes.

Agarose gel dsRNA release study

Polyplexes were formed in sterile water at an N/P ratio of 5 with a final concentration of 1 mg/mL
20 dsRNA. Samples were then divided into separate microtubes for each sample time point and stored at room temperature, until the microtubes were frozen at the appropriate time. When all the time points had been collected, samples were defrosted diluted to 100 µg/mL dsRNA. Prior to loading, loading buffer was added to each sample and 10 µL of polyplexes were loaded into the agarose gel wells. Gel electrophoresis was performed at 100 V for 20 minutes on a 1% agarose gel containing ethidium
25 bromide; results are shown in Figure 2.

Example 4. RNAi stabilisation and release in soil (Agarose Electrophoresis)

Polyplex soil stability assay

Polyplexes were formed in sterile water at an N/P ratio of 5 with a final concentration of 1 mg/mL dsRNA. 200 µL of polyplexes were mixed with 0.5 g soil (live soil containing enzymes, and also
30 sterilised soil (sterilisation conditions: 200°C, 2 hr)) in 2 mL microtubes. Separate microtubes were used for each sample time point and stored at room temperature. At the appropriate time, the reaction was stopped by addition of 1 mL trireagent, vortexing, and incubating for 5 minutes, before storing

the sample time point at -20°C. dsRNA was extracted from the soil in order to analyse the dsRNA by agarose gel electrophoresis. Controls were included of polyplex incubated in sterile water followed by the dsRNA extraction protocol, and also polyplex incubated in sterile water then direct agarose gel electrophoresis without the dsRNA extraction protocol. Controls of dsRNA (without polymer) incubated in live soil, enzyme-free soil, and sterile water, followed by the dsRNA extraction protocol, were included.

dsRNA extraction

Polyplex/soil/trireagent samples were defrosted, 200µL of chloroform added, and incubated for 3 minutes at room temperature. Samples were then centrifuged for 15 minutes at 12000g and 4°C.

Supernatant was added to new microtube, isopropanol added (1/1 ratio) to precipitate the RNA, and incubated for 10 minutes at room temperature. Microtubes were then centrifuged at 12000 g and 4 °C for 10 minutes. Supernatant was removed and 500 µL of 70% ethanol (in RNase free water) added to the pellet, then centrifuged for 5 minutes at 12000 g and 4°C. The subsequent supernatant was removed and the pellet left to dry for 10 minutes before being suspended in 200 µL of RNase free water. These RNA samples were then enriched for dsRNA following a LiCl purification protocol. LiCl (8M, 67µL) was added to the 200µL RNA samples, which were mixed on ice, and incubated for 30 minutes at -20°C. Microtubes were then centrifuged for 20 minutes at 14000g and 4°C, the supernatant was brought to a new microtube, and LiCl (8M, 133.5µL) was added. The samples were mixed on ice, incubated for 30 minutes at -20°C, and then centrifuged for 20 minutes at 14000 g and 4°C. The supernatant was removed, the dsRNA pellet washed with 70% ethanol (in RNase free water, 150 µL), and then centrifuged for 5 minutes at 12000g and 4°C. The subsequent supernatant was removed and the dsRNA pellet left to dry for 5 minutes before being suspended in 20 µL of RNase free water. These final dsRNA samples were analysed by spectrophotometry (NanoPhotometer NP60 spectrophotometer), and agarose gel electrophoresis. Gel electrophoresis was performed at 100 V for 20 minutes on a 1% agarose gel (1x TAE buffer) containing ethidium bromide.

Agarose gel electrophoresis

Agarose gels (1% w/v) were prepared with agarose and 1 × Tris-Acetate-EDTA (TAE) buffer with DNase/RNase free water. The solution was cooled on the bench for 5 minutes and 100µL of 0.5µg/mL ethidium bromide solution was added. The mixture was poured into the casted agarose tray and a comb inserted. The gel was left to set for a minimum of 30 minutes at room temperature. The agarose gels were run in 1× TAE buffer. The final gel was visualized under UV illumination at 365nm using a UVP benchtop UV transilluminator system. Polyplexes of dsRNA were prepared at various N/P ratios. dsRNA stock solution of 60µg/mL was prepared in PBS, and polymer stock solution of

300 µg/mL. For polyplex formation: appropriate amount of polymer stock solution and dsRNA stock solution were mixed and made up to a total volume of 100 µL in PBS (final concentration of dsRNA was 0.030 µg/µL, in all solutions). Polyplexes were vortexed and incubated at room temperature for 30 minutes. Prior to loading, 30 µL of loading buffer was added to each sample and 20 µL of polyplexes were loaded into the agarose gel wells. Gel electrophoresis was performed at 100V for 30 minutes; results are shown in Figure 3.

Example 5. Polymer Cytotoxicity

Cell culture

NIH-3T3 mouse endothelial cells were obtained from the European Collection of Cell Cultures (ECACC) and used between passages 5 and 25, grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% of bovine calf serum, 1% of 2 mM glutamine and 1% penicillin/streptomycin. The cells were grown as adherent monolayers at 310 K under a 5% CO₂ humidified atmosphere and passaged at approximately 70–80% confluence.

In vitro growth inhibition assays

The antiproliferative activity of polymers were determined in 3T3 mouse endothelial cells. Briefly, 96-well plates were used to seed 10,000 cells per well. The plates were left to preincubate with drug-free medium at 310 K for 24 hours before adding different concentrations of the compounds to be tested. A polymer exposure period of 24 hours was allowed. The XTT/PMS assay was used to determine cell viability. Cell viabilities were determined as duplicates of triplicates in two independent sets of experiments and their standard deviations were calculated; results are shown in Figure 4.

Example 6. Polymer Synthesis

Polymer synthesis

For a typical polymerisation, with the conditions [BEA]: [PEGA]: [DEGDA]: [CTA]: [ACVA] = 40: 10: 2.5: 1: 0.1, CTA (10.7 mg, 0.0447 mmol), BEA (0.319 g, 1.78 mmol), PEGA (0.214 g, 0.446 mmol), DEGDA (23.9 mg, 0.112 mmol), ACVA (1.25 mg, 0.00447 mmol), and dioxane (0.445 mL) were added to a vial deoxygenated by bubbling with nitrogen and left to stir in an oil bath at 70 °C. After 24 hours, the solution was removed from the oil bath and the polymer precipitated in diethyl ether (x3), and dried under vacuum. $M_w = 127,900$ g/mol, $D = 5.4$ (DMF SEC, +NH₄BF₄ additive eluent, PMMA calibration). ¹H NMR spectrum (400 MHz, DMSO-*d*₆, δ ppm): 4.34 (m, 2H, -C(O)O-CH₂-CH₂-Br), 4.12 (m, 2H, -C(O)O-CH₂-CH₂-O-), 3.65 (m, 2H, -C(O)O-CH₂-CH₂-Br), 3.52 (m, 32H, -CH₂- (PEG)), 3.24 (s, 3H, -(CH₂-CH₂-O)-CH₃), 2.39–1.54 (m, 3H, backbone), 0.89 (t, 3H, -

CH_3 (CTA)). ^{13}C NMR spectrum (100 MHz, $\text{DMSO}-d_6$, δ ppm): 173.79 (-C(O)O-), 70.26 (-CH₂- (PEG)), 66.82 (-C(O)O-CH₂-), 30.82 (-CH₂-Br), 41.23 (backbone tertiary), 21.87 (backbone -CH₂-). FTIR ν cm⁻¹: 2867 (medium, C-H alkane), 1728 (strong, C=O ester), 1447 (medium, C-H alkane), 1093 (strong, C-O-C PEG). 569 (weak, C-Br). Elemental analyses are shown in Table 1.

- 5 In order to further characterise the branched nature of the synthesised polymers, two linear p(BEA-co-PEGA) polymers were synthesised to give a comparison for the KMHS intrinsic viscosity vs molecular weight plots, and associated α values. The polymerisation conditions were identical to the above branched polymers, but without DEGDA crosslinker (conditions and characterisation data can be seen in Table 1). $M_n = 9,700$ g/mol, $\bar{D} = 1.12$; and $M_n = 16,800$ g/mol, $\bar{D} = 1.32$; (DMF SEC, +NH₄BF₄ additive eluent, PMMA calibration). ^1H NMR spectrum (400 MHz, $\text{DMSO}-d_6$, δ ppm): 4.34 (m, 2H, -C(O)O-CH₂-CH₂-Br), 4.12 (m, 2H, -C(O)O-CH₂-CH₂-O-), 3.65 (m, 2H, -C(O)O-CH₂-CH₂-Br), 3.52 (m, 32H, -CH₂- (PEG)), 3.24 (s, 3H, -(CH₂-CH₂-O)-CH₃), 2.39–1.54 (m, 3H, backbone), 0.89 (t, 3H, -CH₃ (CTA)). ^{13}C NMR spectrum (100 MHz, $\text{DMSO}-d_6$, δ ppm): 174.90 (-C(O)O-), 70.19 (-CH₂- (PEG)), 65.52 (-C(O)O-CH₂-), 31.32 (-CH₂-Br), 41.28 (backbone tertiary), 22.87 (backbone -CH₂-). FTIR ν cm⁻¹: 2880 (medium, C-H alkane), 1725 (strong, C=O ester), 1440 (medium, C-H alkane), 1095 (strong, C-O-C PEG), 576 (weak, C-Br).

Table 1 Polymerisation conditions for both branched and linear BEA, PEGA copolymers and characterisation data from SEC and ^1H NMR spectroscopic analysis.

Polymer	Conditions [BEA]:[PEGA]:[DEGDA] :[CTA]:[I]	DRI SEC			TD SEC		^1H NMR		
		M_n (g/mol)	M_w (g/mol)	$\bar{D}$	M_w (g/mol)	α	DP BEA PEGA		M_n (g/mol)
Branched	40:10:2.5:1:0.1	23,600	127,900	5.41	220,900	0.35	58	17	-
Linear	64:16:0:1:0.1	9,700	10,800	1.12	13,900	0.6	39	9	11,300
Linear	240:60:0:1:0.1	16,800	22,100	1.32	31,800	0.78	160	35	45,400

20 Post-polymerisation modification

Typical post-polymerization substitution of branched p(BEA-co-PEGA) with trimethylamine: p(BEA-co-PEGA) (0.10g of polymer, 0.447mmol of BEA units) was dissolved in 2 mL of DMSO in a small vial with a stirrer bar, to which was added 2.5 equiv. of trimethylamine (4.2 M in ethanol, 266 μ L, 1.12mmol) and stirred for 48 h under a nitrogen atmosphere. Upon completion, the solution was

concentrated by nitrogen flow, purified by precipitation into THF, and dried under vacuum, to give the desired p(TMAEA-co-PEGA). ^1H NMR (400 MHz, DMSO- d_6 , ppm): δ = 4.53 (m, 2H, -C(O)O-CH $_2$ -CH $_2$ -NMe $_3$), 4.12 (m, 2H, -C(O)O-CH $_2$ -CH $_2$ -O-), 3.91 (m, 2H, -C(O)O-CH $_2$ -CH $_2$ -NMe $_3$), 3.52 (m, 32H, -CH $_2$ - (PEG)), 3.34 (m, 9H, CH $_2$ -CH $_2$ -NMe $_3$), 3.24 (s, 3H, -(CH $_2$ -CH $_2$ -O)-CH $_3$), 2.41–1.51 (m, 3H, backbone), 0.89 (m, 3H, -CH $_3$ (CTA)). ^{13}C NMR spectrum (100 MHz, DMSO- d_6 , δ ppm): 174.04 (-C(O)O-), 70.22 (-CH $_2$ - (PEG)), 64.00 (-C(O)O-CH $_2$ -CH $_2$ -), 58.53 (-C(O)O-CH $_2$ -CH $_2$ -), 53.42 (-N(CH $_3$) $_3$), 40.85 (backbone tertiary), 27.02 (backbone -CH $_2$ -). FTIR ν cm $^{-1}$: 2871 (medium, C-H alkane), 1728 (strong, C=O ester), 1477 (medium, C-H alkane), 1248 (medium, C-N, amine), 1092 (strong, C-O-C PEG). Elemental analyses are shown in Table 2.

Typical post-polymerization substitution of branched p(BEA-co-PEGA) with trimethylphosphine: p(BEA-co-PEGA) (0.10g of polymer, 0.447mmol of BEA units) was dissolved in 2mL of DMSO in a small vial with a stirrer bar, to which was added 2.5 equiv. of trimethylphosphine (1M in THF, 1.12mL, 1.12mmol) and stirred for 48 h under a nitrogen atmosphere. Upon completion, the solution was concentrated by nitrogen flow, purified by precipitation into THF, and dried under vacuum, to give the desired p(TMPEA-co-PEGA). ¹H NMR (300 MHz, DMSO-*d*₆, ppm): δ = 4.36 (m, 2H, -C(O)O-CH₂-CH₂-PMe₃), 4.13 (m, 2H, -C(O)O-CH₂-CH₂-O-), 3.51 (m, 32H, -CH₂- (PEG)), 3.24 (s, 3H, -(CH₂-CH₂-O)-CH₃), 2.83 (m, 2H, -C(O)O-CH₂-CH₂-PMe₃), 2.07 (m, 9H, CH₂-CH₂-PMe₃), 2.46–1.44 (m, 3H, backbone), 1.06 (m, 3H, -CH₃ (CTA)). ¹³C NMR spectrum (100 MHz, DMSO-*d*₆, δ ppm): 174.13 (-C(O)O-), 70.23 (-CH₂- (PEG)), 60.64 (-C(O)O-CH₂-CH₂-), 54.87 (-C(O)O-CH₂-CH₂-), 40.55 (backbone tertiary), 26.82 (backbone -CH₂-), 5.30 (-P(CH₃)₃). FTIR ν cm⁻¹: 2897 (medium, C-H alkane), 1728 (strong, C=O ester), 1420 (medium, C-H alkane), 1087 (strong, C-O-C PEG), 971 (strong, P-CH₃). Elemental analyses are shown in Table 2.

Figure 5 has ^1H NMR spectra before and after substitution.

Table 2. Elemental analysis results for branched BEA PEGA copolymer, and post-polymerisation modified polymers, branched p(TMPEA-co-PEGA) and p(TMAEA-co-PEGA); calculated (Calc.) vs. Measured (Msd).

Polymer	C (%)		H (%)		N (%)		Br (%)		S (%)	
	Calc.	Msd.	Calc.	Msd.	Calc.	Msd.	Calc.	Msd.	Calc.	Msd.

Branched p(BEA-co-PEGA) $C_{445.5}H_{739}$ $Br_{40}O_{198.25}S_3$	42.60	41.95	5.93	6.08	0.00	0.00	25.45	24.91	0.77	1.01
Branched p(TMAEA-co-PEGA) $C_{565.5}H_{1099}$ $Br_{40}N_{40}O_{198.25}S_3$	45.51	40.45	7.42	7.79	3.75	3.10	21.42	19.88	0.64	1.99
Branched p(TMPEA-co-PEGA) $C_{565.5}H_{1099}$ $Br_{40}O_{198.25}P_{40}S_3$	43.53	38.04	7.10	7.22	0.00	0.00	20.48	19.21	0.62	1.10

Example 7. Polymer Hydrolysis

The hydrolysis kinetics of the branched polyphosphonium polymers was studied using 1H NMR spectroscopy in D_2O . Polymers (10 mg) were dissolved in D_2O (0.6 mL, pH ~ 7.4), incubated at room temperature, and 1H NMR spectra then acquired over a period of 4 weeks. No evidence of hydrolysis was observed after 4 weeks in D_2O ; see Figure 6.

Example 8. RNAi Complexation and Release into Water (Agarose Electrophoresis)

Agarose gel electrophoresis

- Agarose gels (1% w/v) were prepared with agarose and $1 \times$ TAE buffer. The solution was cooled on the bench for 5 minutes and 100 μ L of 0.5 μ g/mL ethidium bromide solution was added. The mixture was poured into the casted agarose tray and a comb inserted. The gel was left to set for a minimum of 30 minutes at room temperature. The agarose gels were run in $1 \times$ TAE buffer. The final gel was visualized under UV illumination at 365 nm using a UVP benchtop UV transilluminator system.
- Polyplexes of DNA were prepared at various N/P ratios. DNA stock solution of 60 μ g/mL was prepared in PBS, and polymer stock solution of 300 μ g/mL. For polyplex formation: appropriate amount of polymer stock solution and DNA stock solution were mixed and made up to a total volume of 100 μ L in PBS (final concentration of DNA was 0.030 μ g/ μ L, in all solutions). Polyplexes were vortexed and incubated at room temperature for 30 minutes. Prior to loading, 30 μ L of loading buffer

was added to each sample and 20 μ L of polyplexes were loaded into the agarose gel wells. Gel electrophoresis was performed at 100 V for 30 minutes. Results are provided in Figure 7.

Example 9. RNAi stabilisation and release in soil (Agarose Electrophoresis)

5 Polyplex soil stability assay

Polyplexes were formed in sterile water at an N/P ratio of 5 with a final concentration of 1 mg/mL dsRNA. 200 μ L of polyplexes were mixed with 0.5 g soil (live soil containing enzymes, and also sterilised soil (sterilisation conditions: 200 °C, 2 hr)) in 2 mL microtubes. Separate microtubes were used for each sample time point and stored at room temperature. At the appropriate time, the reaction
10 was stopped by addition of 1 mL trireagent, vortexing, and incubating for 5 minutes, before storing the sample time point at -20 °C. dsRNA was extracted from the soil in order to analyse the dsRNA by agarose gel electrophoresis. Controls were included of polyplex incubated in sterile water followed by the dsRNA extraction protocol, and also polyplex incubated in sterile water then direct agarose gel electrophoresis without the dsRNA extraction protocol. Controls of dsRNA (without polymer)
15 incubated in live soil, and enzyme-free soil, followed by the dsRNA extraction protocol, were included.

dsRNA extraction

Polyplex/soil/trireagent samples were defrosted, 200 μ L of chloroform added, and incubated for 3 minutes at room temperature. Samples were then centrifuged for 15 minutes at 12000 g and 4 °C.
20 Supernatant was added to new microtube, isopropanol added (1/1 ratio) to precipitate the RNA, and incubated for 10 minutes at room temperature. Microtubes were then centrifuged at 12000 g and 4 °C for 10 minutes. Supernatant was removed and 500 μ L of 70 % ethanol (in RNase free water) added to the pellet, then centrifuged for 5 minutes at 12000 g and 4 °C. The supernatant was removed and the pellet left to dry for 10 minutes before being suspended in 200 μ L of RNase free water. These RNA
25 samples were then enriched for dsRNA following a LiCl purification protocol. LiCl (8M, 67 μ L) was added to the 200 μ L RNA samples, which were mixed on ice, and incubated for 30 minutes at -20 °C. Microtubes were then centrifuged for 20 minutes at 14000 g and 4 °C, the supernatant was brought to a new microtube, and LiCl (8M, 133.5 μ L) was added. The samples were mixed on ice, incubated for 30 minutes at -20 °C, and then centrifuged for 20 minutes at 14000 g and 4 °C. The supernatant was
30 removed, the dsRNA pellet washed with 70% ethanol (in RNase free water, 150 μ L), and then centrifuged for 5 minutes at 12000 g and 4 °C. The supernatant was removed and the dsRNA pellet left to dry for 5 minutes before being suspended in 20 μ L of RNase free water. These final dsRNA samples were analysed by spectrophotometry (NanoPhotometer NP60 spectrophotometer), and

agarose gel electrophoresis. Gel electrophoresis was performed at 100 V for 20 minutes on a 1% agarose gel (1x TAE buffer) containing ethidium bromide.

Agarose gel electrophoresis

Agarose gels (1% w/v) were prepared with agarose and 1 × Tris-Acetate-EDTA (TAE) buffer with DNase/RNase free water. The solution was cooled on the bench for 5 minutes and 100 µL of 0.5 µg/mL ethidium bromide solution was added. The mixture was poured into the casted agarose tray and a comb inserted. The gel was left to set for a minimum of 30 minutes at room temperature. The agarose gels were run in 1× TAE buffer. The final gel was visualized under UV illumination at 365 nm using a UVP benchtop UV transilluminator system. Polyplexes of dsRNA were prepared at various N/P ratios. dsRNA stock solution of 60 µg/mL was prepared in PBS, and polymer stock solution of 300 µg/mL. For polyplex formation: appropriate amount of polymer stock solution and dsRNA stock solution were mixed and made up to a total volume of 100 µL in PBS (final concentration of dsRNA was 0.030 µg/µL, in all solutions). Polyplexes were vortexed and incubated at room temperature for 30 minutes. Prior to loading, 30 µL of loading buffer was added to each sample and 20 µL of polyplexes were loaded into the agarose gel wells. Gel electrophoresis was performed at 100 V for 30 minutes. Results are provided in Figure 8.

Example 10. Polymer Cytotoxicity

Cell culture

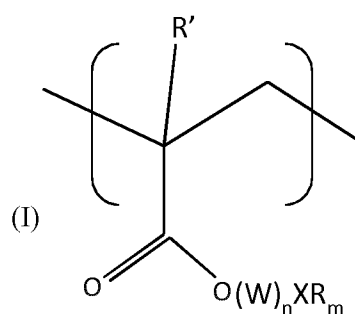
3T3 mouse endothelial cells were obtained from the European Collection of Cell Cultures (ECACC) and used between passages 5 and 25, grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% of fetal calf serum, 1% of 2 mM glutamine. The cells were grown as adherent monolayers at 310 K under a 5% CO₂ humidified atmosphere and passaged at approximately 70–80% confluence.

In vitro growth inhibition assays

The antiproliferative activity of polymers were determined in NIH-3T3 mouse endothelial cells. Briefly, 96-well plates were used to seed 10,000 cells per well. The plates were left to preincubate with drug-free medium at 310 K for 24 h before adding different concentrations of the compounds to be tested. A polymer exposure period of 24 h was allowed. The XTT/PMS assay was used to determine cell viability. Cell viabilities were determined as duplicates of triplicates in two independent sets of experiments and their standard deviations were calculated; see Figure 9.

CLAIMS

1. A composition comprising a polymer comprising monomer units of formula (I) and monomer units of formula (II); and a single dsRNA sequence or a mixture of dsRNA sequences:



10 where

$R' = \text{H or } \text{CH}_3$;

W is CH_2 or $\text{CH}_2\text{CH}_2\text{O}$;

when W is CH_2 then n is an integer from 2 to 5;

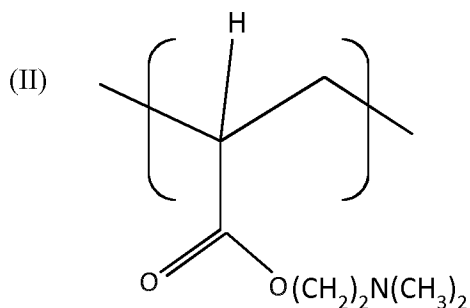
when W is $\text{CH}_2\text{CH}_2\text{O}$ then n is an integer from 1 to 100;

15 X is N, N^+ , P or P^+ ;

m is 2 when X is N or P; m is 3 when X is N^+ or P^+ ; and

each R is independently a linear or branched C_{1-6} alkyl group or, when X is N, then XR_m may be a saturated ring comprising up to six carbon atoms and up to a further two heteroatoms each independently selected from O and N;

20 provided that R' is CH_3 when W is CH_2 , n is 2, X is N and R is CH_3 ;



2. A composition as claimed in claim 1, wherein the polymer has an average M_n greater than 2 kDa.
- 5 3. A composition as claimed in claim 1 or 2 where the numerical ratio of units of formula (I) to units of formula (II) is from 1:99 to 99:1.
4. A composition as claimed in any one of claims 1-3 where the polymer has a linear, star, dendrimer, branched or hyperbranched architecture.
- 10 5. A composition as claimed in any one of claims 1-4 where the composition is a suspension concentrate, a wettable powder or a water dispersible granule.
6. A composition as claimed in any one of claims 1-4 where the composition is a suspension concentrate, wherein polymer composition is from 0.2% to 20% of the total weight and dsRNA is from 0.1% to 5% of the total weight of the composition.
7. A composition as claimed in any one of claims 1-4 where the composition is a wettable powder or a water dispersible granule wherein polymer composition is from 1% to 80% of the total weight and dsRNA is from 0.5% to 20% of the total weight of the composition.
- 15 8. A composition as claimed in any one of the preceding claims which further comprises at least one agronomically acceptable excipient and/or diluent, and optionally at least one pesticidally active ingredient.
9. Use of a composition as claimed in any one of the preceding claims for controlling pest growth and/or plant infestation.
- 20 10. A method of substantially retaining or otherwise preserving the biological activity of a dsRNA present in a degradative environment, comprising complexing a single dsRNA or mixture of dsRNA sequences and a polymer as defined in any one of claims 1-4.
11. A method as claimed in claim 10 where the polymer controls the rate of hydrolysis and thus release of dsRNA.
- 25 12. A method as claimed in claim 10 or 11, wherein said dsRNA comprises a strand that is complementary to at least part of a nucleotide sequence of a gene from a plant pest organism
13. A method as claimed in claim 10, 11 or 12 where the dsRNA is from 15 to 1800 base pairs in length.

14. A method of controlling a plant pest infestation, preferably a subterranean plant pest, comprising:

- (a) providing a composition as claimed in any one of claims 1 to 8;
- (b) applying the composition to the locus of a plant, preferably being soil; and
- (c) releasing the dsRNA from the composition in the locus by hydrolysis of the polymer.

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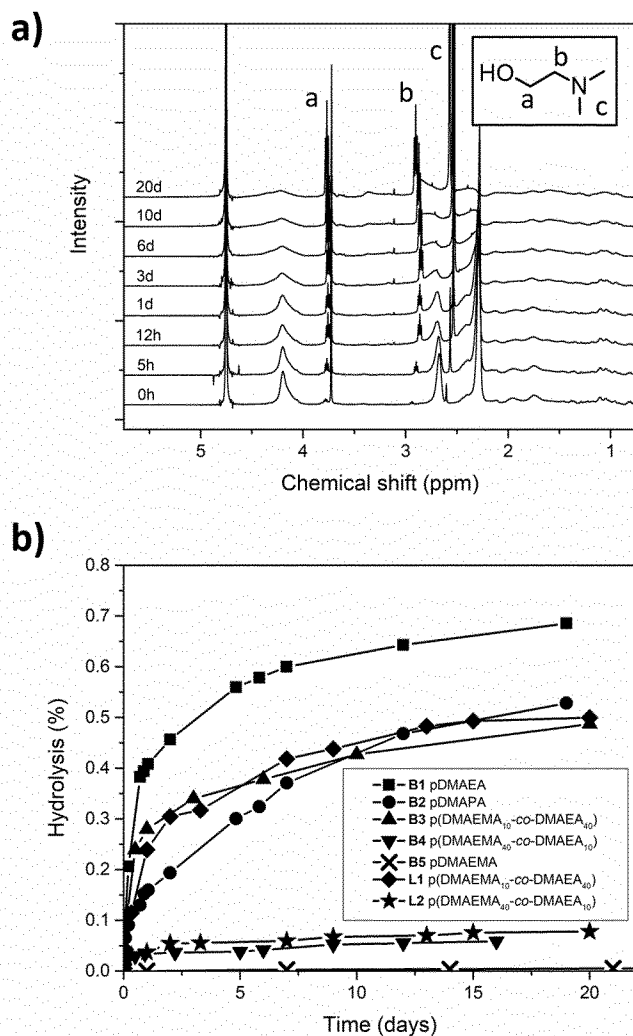


Figure 1 a) Example of the hydrolysis of branched $p(\text{DMAEMA}_{10}\text{-co-DMAEA}_{40})$ in D_2O ($\text{pH} \sim 7.4$) determined using ^1H NMR spectroscopy; **b)** Hydrolysis kinetics of synthesised series of tertiary amine containing branched and linear polymers in D_2O ($\text{pH} \sim 7.4$) determined using ^1H NMR spectroscopy.

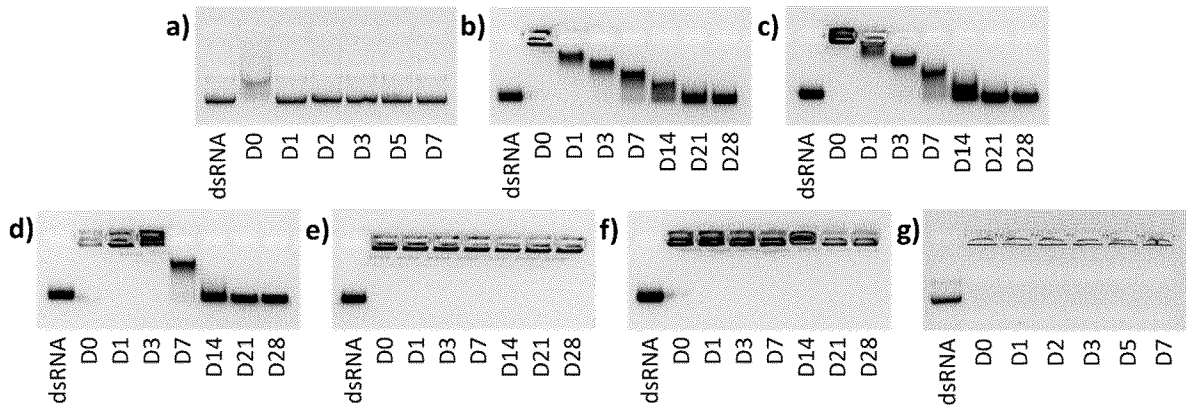


Figure 2. Agarose gel electrophoresis assay to assess dsRNA release from branched polymer polyplex nanoparticles (all at N/P ratio 5) over time periods up to 28 days; dsRNA as control, **a)** branched p(DMAEA); **b)** linear p(DMAEMA₁₀-co-DMAEA₄₀); **c)** branched p(DMAEMA₁₀-co-DMAEA₄₀); **d)** branched p(DMAPA); **e)** linear p(DMAEMA₄₀-co-DMAEA₁₀); **f)** branched p(DMAEMA₄₀-co-DMAEA₁₀); **g)** branched p(DMAEMA).

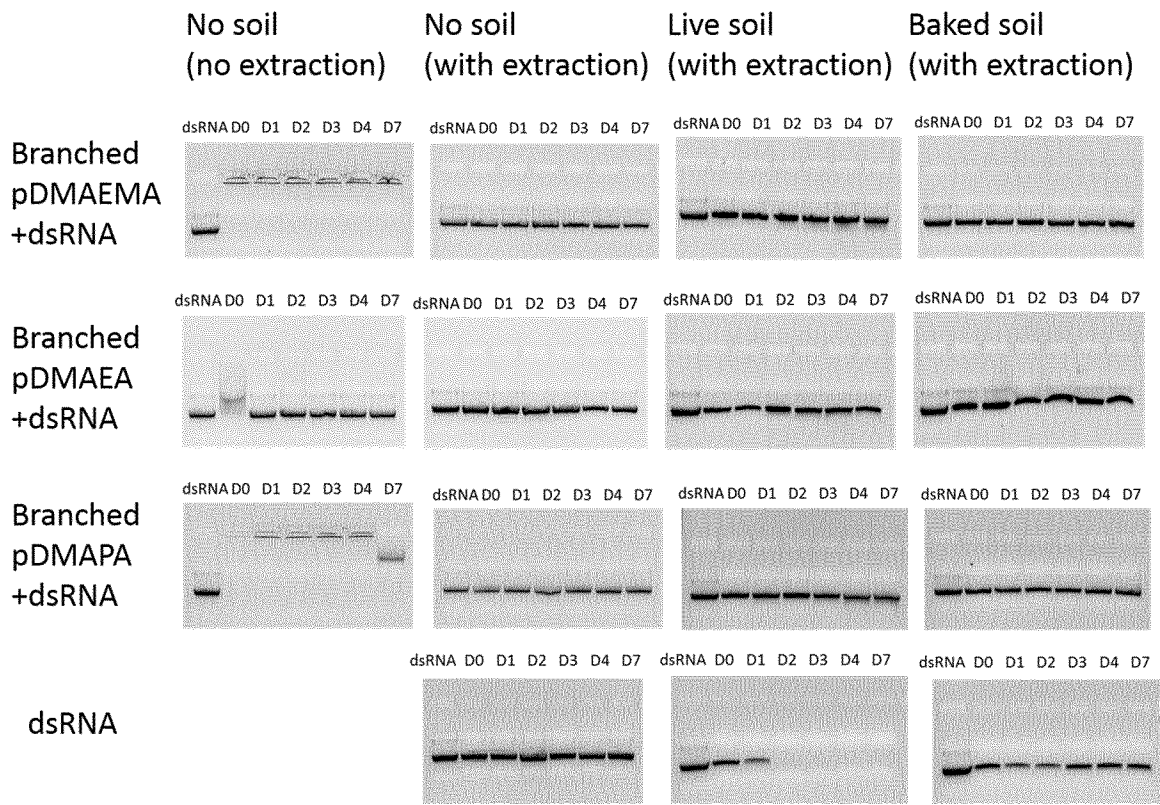


Figure 3. Polyplex soil stability assay (dsRNA protection), polyplexes were formed in sterile water at an N/P ratio of 5 with a final concentration of 1 mg/mL dsRNA, incubated in live soil or enzyme free soil, then dsRNA was extracted and run on an agarose gel. Control dsRNA soil stability assay, with a final concentration of 1 mg/mL dsRNA, incubated in live soil or enzyme free soil, then dsRNA was extracted and run on an agarose gel.

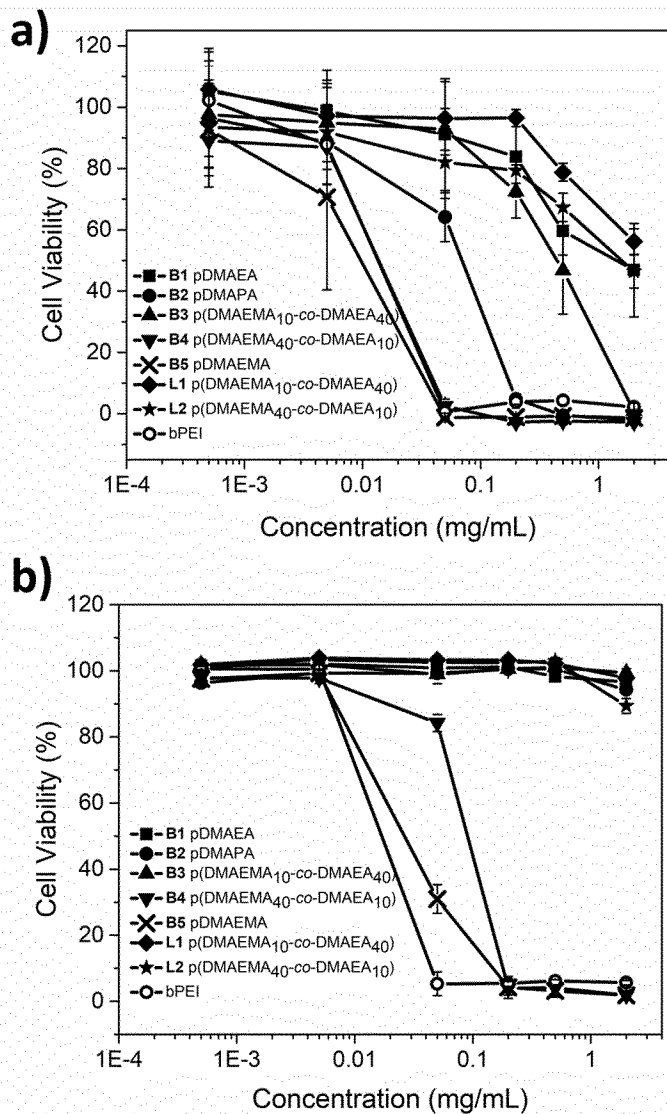


Figure 4. Cell viability assay for the synthesised series of linear and highly branched polymers (XTT assay) with 3T3 fibroblast cell-line after 24 hr incubation; **a)** Initial non hydrolysed tertiary amine containing polymers; **b)** hydrolysed polymers after 2 weeks incubation in D2O at pH ~7.4.

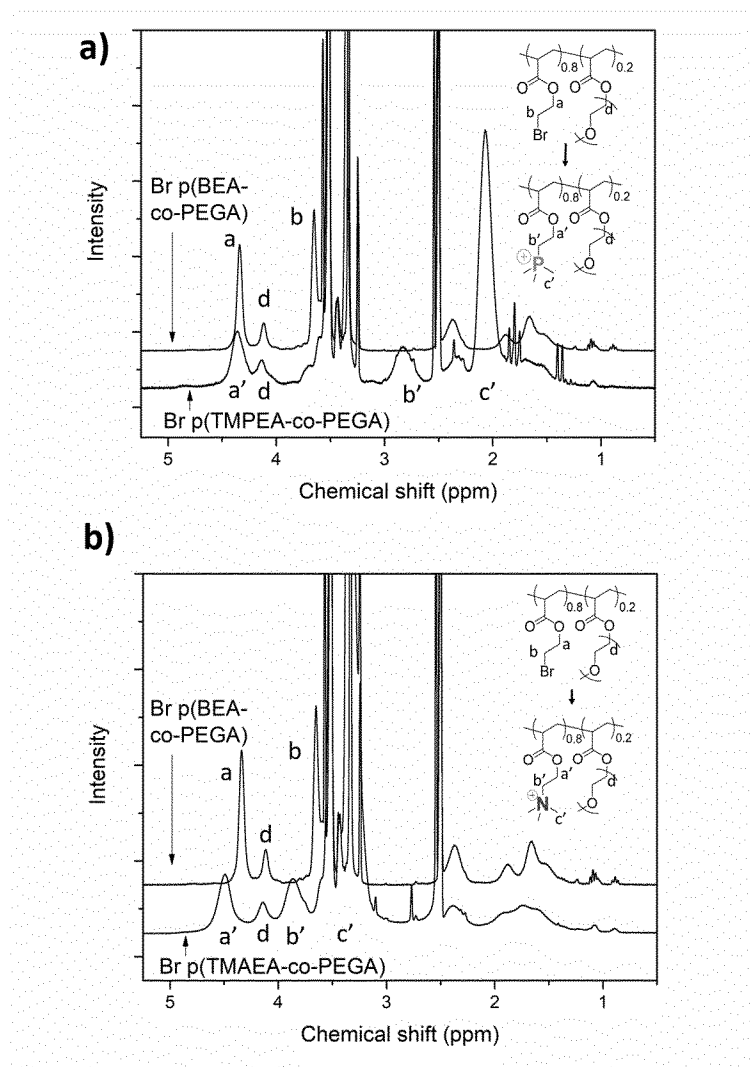


Figure 5. a) ^1H NMR spectra in DMSO-d_6 of branched p(BEA-co-PGA) before and after substitution to form p(TMPEA-co-PGA) indicating the shifts of the pendant ethyl acrylate chain protons; **b)** ^1H NMR spectra in DMSO-d_6 of branched p(BEA-co-PGA) before and after substitution to form p(TMAEA-co-PGA) indicating the shifts of the pendant ethyl acrylate chain protons.

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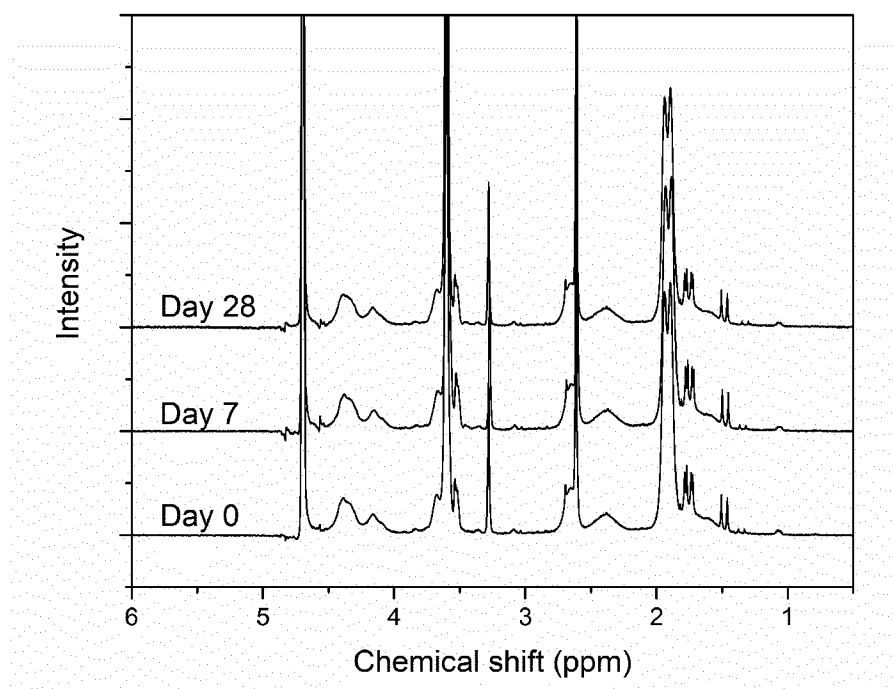


Figure 6. Proton (¹H) NMR spectra of branched p(TMPEA-co-PEGA) in D₂O over 4 weeks at room temperature and pH 7.

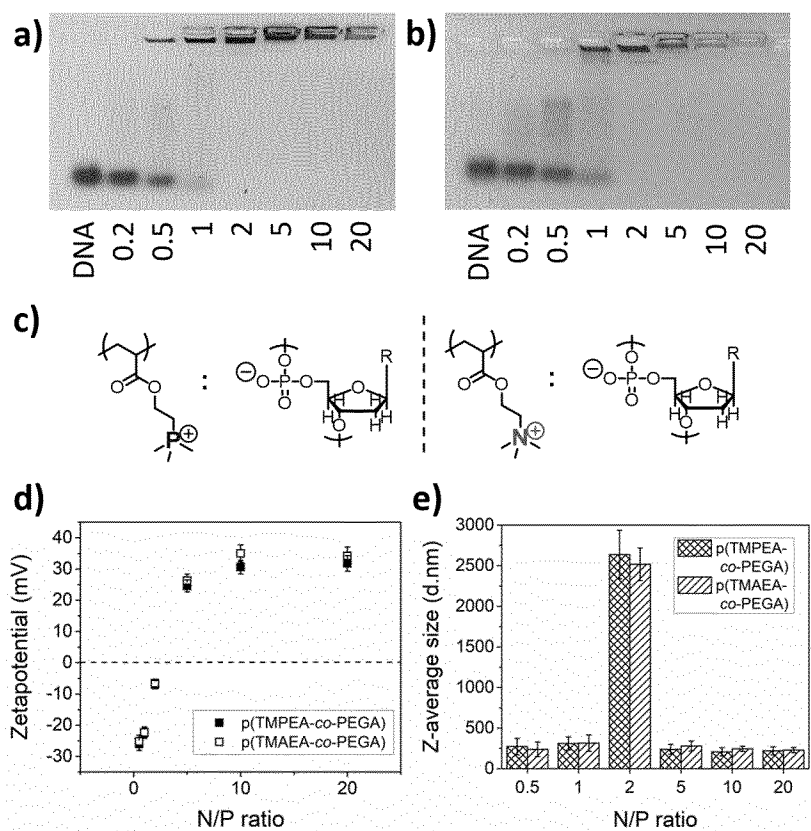


Figure 7 **a)** p(TMPEA-co-PEGA) polyplex formation with DNA characterised by agarose gel electrophoresis at varying P⁺/P charge ratios; **b)** p(TMAEA-co-PEGA) polyplex formation with DNA characterised by agarose gel electrophoresis at varying N⁺/P charge ratios; **c)** structures of repeating units for N⁺/P and P⁺/P charge ratios; **d)** surface charge (zetapotential) and **e)** size of polyplexes formed with DNA at varying N⁺/P or P⁺/P ratios measured by dynamic light scattering and electrophoretic light scattering.

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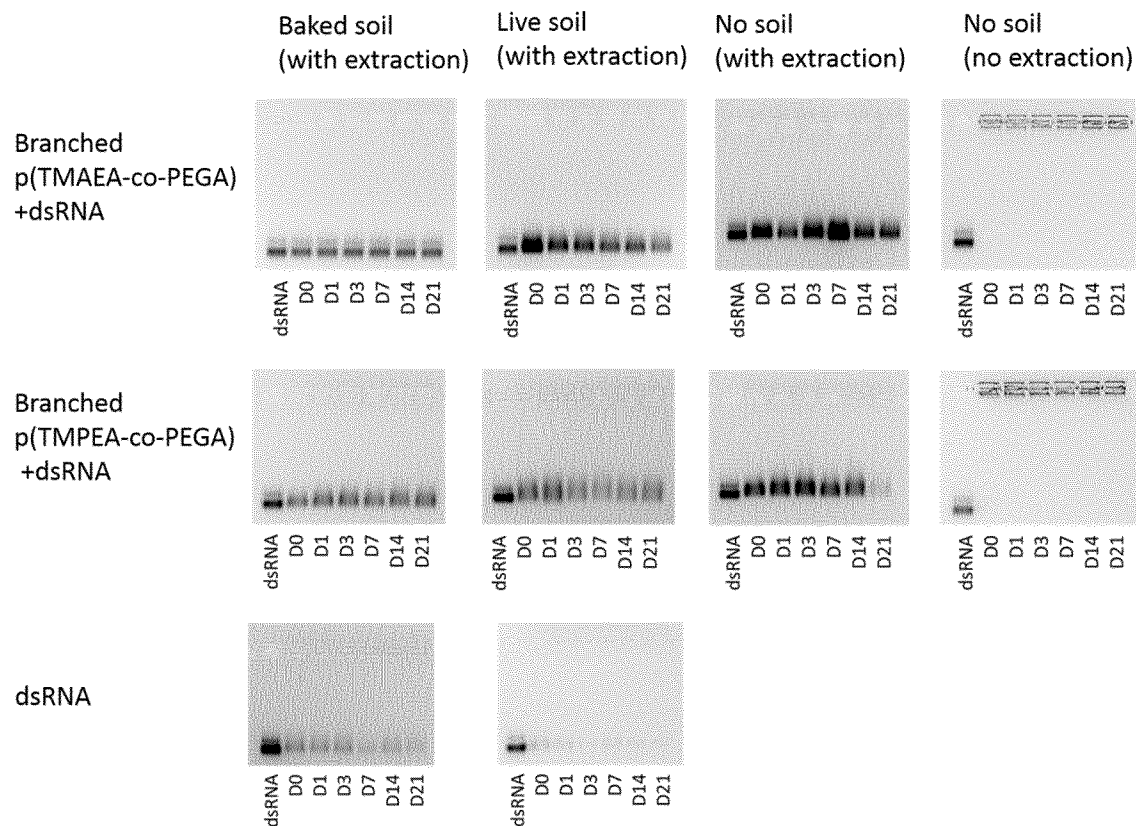


Figure 8. Polyplex soil stability assay (dsRNA protection), polyplexes were formed in sterile water at an N/P or P/P ratio of 5 with a final concentration of 1 mg/mL dsRNA, incubated in live soil or enzyme free soil, then dsRNA was extracted and run on an agarose gel.

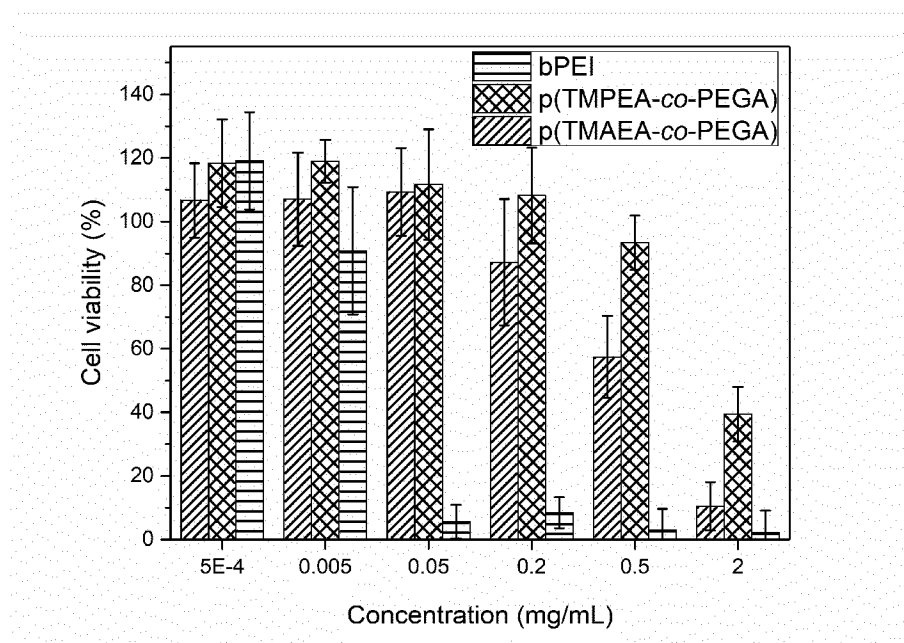


Figure 9. Cell viability of 3T3 fibroblast cells treated with branched p(TMPEA-co-PEGA), branched p(TMAEA-co-PEGA), and bPEI for 24 hours at 37°C.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/062939

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	A01N25/08 A01P7/00	A01N25/22 A01N57/16 C12N15/11 C12N15/113
ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) A01N C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, WPI Data, CHEM ABS Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2016/201523 A1 (UNIV QUEENSLAND [AU]) 22 December 2016 (2016-12-22) cited in the application	1-14
Y	the claims; the examples and paragraphs 3-22, 24-61, 73-76, 82-92, 98-103 and 125-129	1-14
X	US 2015/024488 A1 (GUNATILLAKE PATHIRAJA ARACHCHILLAGE [AU] ET AL) 22 January 2015 (2015-01-22) cited in the application	1-8, 10-13
Y	the claims; the examples, and paragraphs 6-9, 19, 26, 57, 97, 192, 203 and 211	9,14
	----- -/-	
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 21 June 2019		Date of mailing of the international search report 03/07/2019
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Lorenzo Varela, M

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/062939

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	SOARES C A G ET AL: "Capillary feeding of specific dsRNA induces silencing of the isac gene in nymphal Ixodes scapularis ticks", INSECT MOLECULAR BIOLOGY, BLACKWELL SCIENTIFIC, OXFORD, GB, vol. 14, no. 4, 1 August 2005 (2005-08-01), pages 443-452, XP002446932, ISSN: 0962-1075, DOI: 10.1111/J.1365-2583.2005.00575.X page 443; page 444, left column, paragraphs 1 and 2; page 447, the discussion; page 448; page 449, left column, first paragraph -----	8,14
Y	WENYI GU; ZHONGFAN JIA; NGHIA P. TRUONG; INDIRA PRASADAM; YIN XIAO; MICHAEL J. MONTEIRO: "Polymer Nanocarrier System for Endosome Escape and Timed Release of siRNA with Complete Gene Silencing and Cell Death in Cancer Cells", BIOMACROMOLECULES, vol. 14, no. 10, 30 August 2013 (2013-08-30), pages 3386-3389, XP009514040, cited in the application pages 3386 and 3387 -----	1-14

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Information on patent family members

International application No

PCT/EP2019/062939

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