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Efficient Binding, Protection, and Self-Release of dsRNA in Soil by Linear and Star Cationic Polymers

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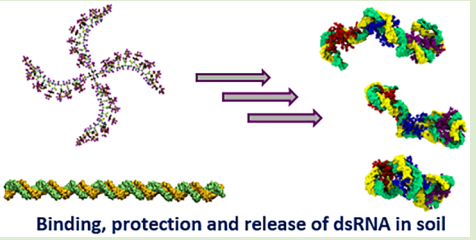
Supporting Information

ABSTRACT: Double stranded RNA (dsRNA) exhibits severe degradation within 3 days in live soil, limiting its potential application in crop protection. Herein we report the efficient binding, protection, and self-release of dsRNA in live soil through the usage of a cationic polymer. Soil stability assays show that linear poly(2-(dimethylamino)ethyl acrylate) can delay the degradation of dsRNA by up to 1 week while the star shaped analogue showed an increased stabilization of dsRNA by up to 3 weeks. Thus, the architecture of the polymer can significantly affect the lifetime of dsRNA in soil. In addition, the hydrolysis and dsRNA binding and release profiles of these polymers were carefully evaluated and discussed. Importantly, hydrolysis could occur independently of environmental conditions (e.g., different pH, different temperature) showing the potential for many opportunities in agrochemicals where protection and subsequent self-release of dsRNA in live soil is required.



RNA interference (RNAi) is a naturally occurring process, where double stranded RNA (dsRNA) can regulate protein expression.[1−3] The use of dsRNA in the agrochemical industry is desirable as selected pests can be specifically targeted, while eliminating the detrimental effects of existing chemical pesticides on nontarget species.[4] This technique is advantageous as an alternative method of chemical control to help mitigate the development of resistance by natural selection and also minimizes potential environmental impact associated with current pest control methods.[5] However, the effectiveness of RNAi is limited by the very short lifetime of dsRNA which is susceptible to degradation under environmental conditions, with numerous pathways reported.[6] Ribonucleases (RNases), for example are enzymes which degrade RNA into smaller fragments, and are not only found within the environment, but also in the air, dust and on surfaces. This inherent instability and short half-life of dsRNA when in contact with these enzymes represents a serious challenge in applying RNAi to agrochemicals. Although most of the reports focus on delivering dsRNA to insects through microinjection into the hemolymph or feeding,[5,7,8] RNAi has also been shown to be effective in knocking down insect genes in plants, with delivery on to the surface of a leaf prior to insect feeding, or through in vivo dsRNA production within the chloroplast.[9−11] Nevertheless, all these methods are challenging for the application to large scale agrochemicals.[5,12] A potential alternative route for delivering dsRNA to the plant could be via soil, followed by uptake through the roots, or ingestion by a pest. It has been illustrated that root cells can absorb dsRNA and RNAi can be triggered;[13,14] however, applying this process to soil creates additional challenges as soil contains many chemicals (salts, minerals and nutrients), enzymes and living (micro)organisms, which can interact and vastly increase the rate of degradation of RNA.[15−17] A method of protecting dsRNA and increasing lifetime in soil would be highly desirable, yet no efforts on stabilizing dsRNA in soil have been reported.

Cationic polymers have been extensively employed to protect RNA and DNA from degradation with numerous natural and synthetic examples[18,19] including amine functionalized polysaccharides,[20] poly(L-lysine),[21] poly(amidoamines),[22] poly(amino-*co*-ester)s,[23,24] poly((dimethylamino)ethyl methacrylate) (PDMAEMA),[25] and poly(ethylene imines).[26] Although these polymers can efficiently bind to RNA they are however incapable of release due to the very high positive charge density. Release must

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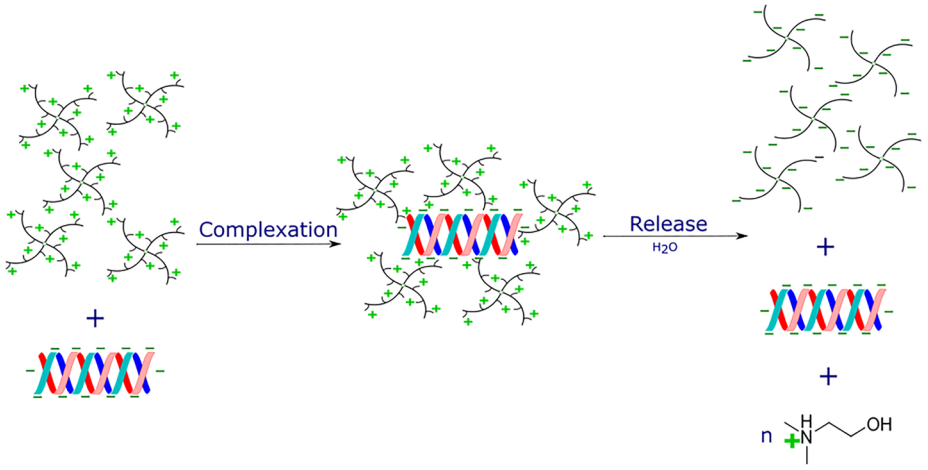
Scheme 1. Schematic Representation of the Complexation of Star PDMAEA to dsRNA and Subsequent Release of dsRNA and the Small Molecule 2-(Dimethylamino)ethanol



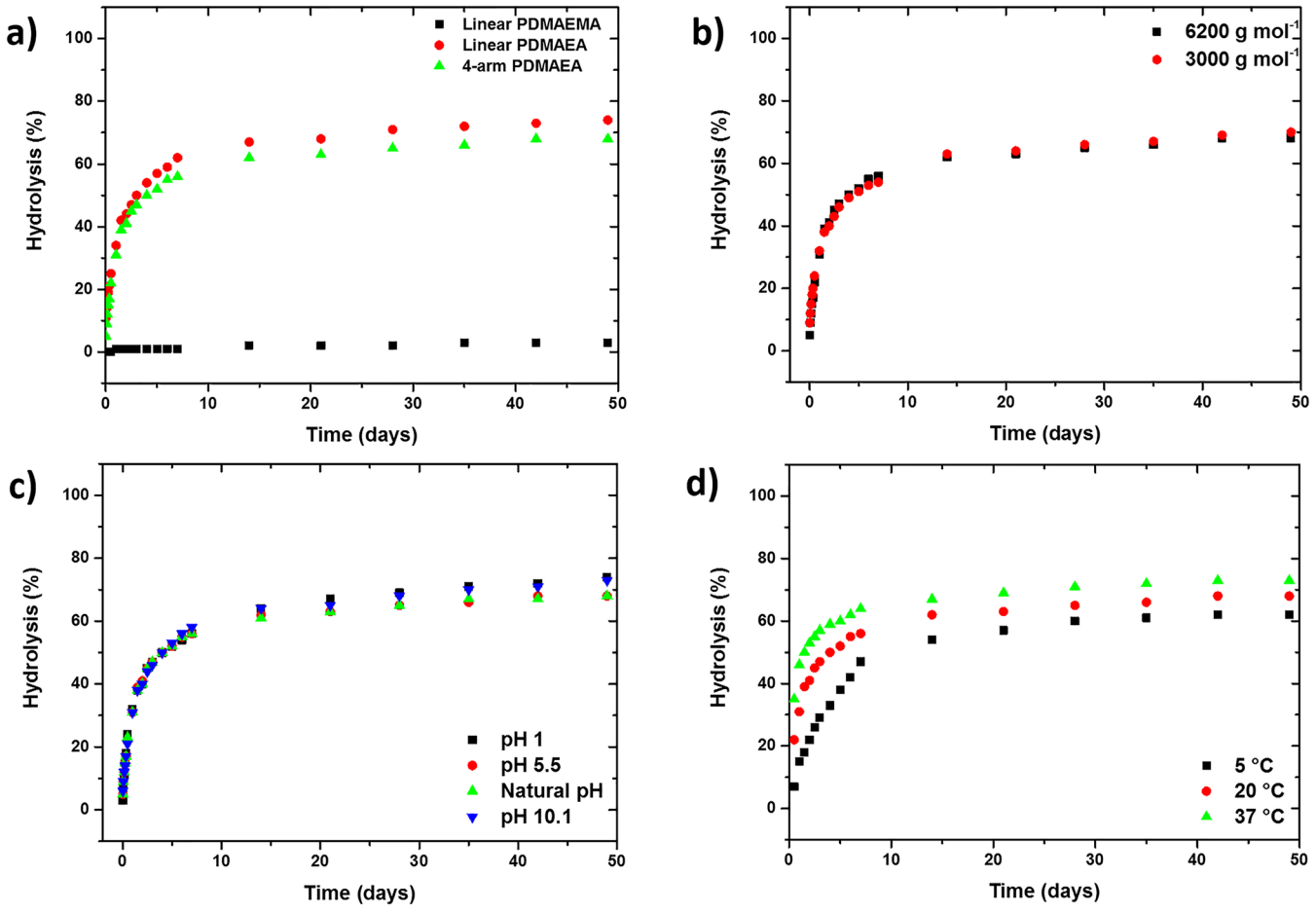


Figure 1. Effect of (a) architecture, (b) molecular weight, (c) temperature, and (d) pH on the rate of hydrolysis of 6200 g mol^{-1} star PDMAEA.

occur to allow the dsRNA to become available so to trigger RNAi.[27] Considerable efforts have been directed at overcoming this problem, in particular, poly(2-(dimethylamino)-ethyl acrylate) (PDMAEA) has been reported as having a self-catalyzed hydrolysis property, with autodegradation to poly-(acrylic acid) and 2-(dimethylamino)ethanol when in aqueous solution.[28−30] In addition, PDMAEA has a high transfection efficiency into HeLa cells when complexed with RNA, can facilitate complete release of RNA and exhibits very low toxicity.[27,31−33] We thus envisaged that PDMAEA could be a good candidate to protect the dsRNA in soil and delay degradation prior to release.

Herein we study for the first time the use of a cationic polymer to increase the lifetime of dsRNA in soil. The effect of the polymerbackbone (poly(acrylate) vs poly-(methacrylate)),[34] polymer architecture (linear vs star), and the soil environment (pH and tempera-ture) on the rate of hydrolysis is thoroughly investigated and discussed.

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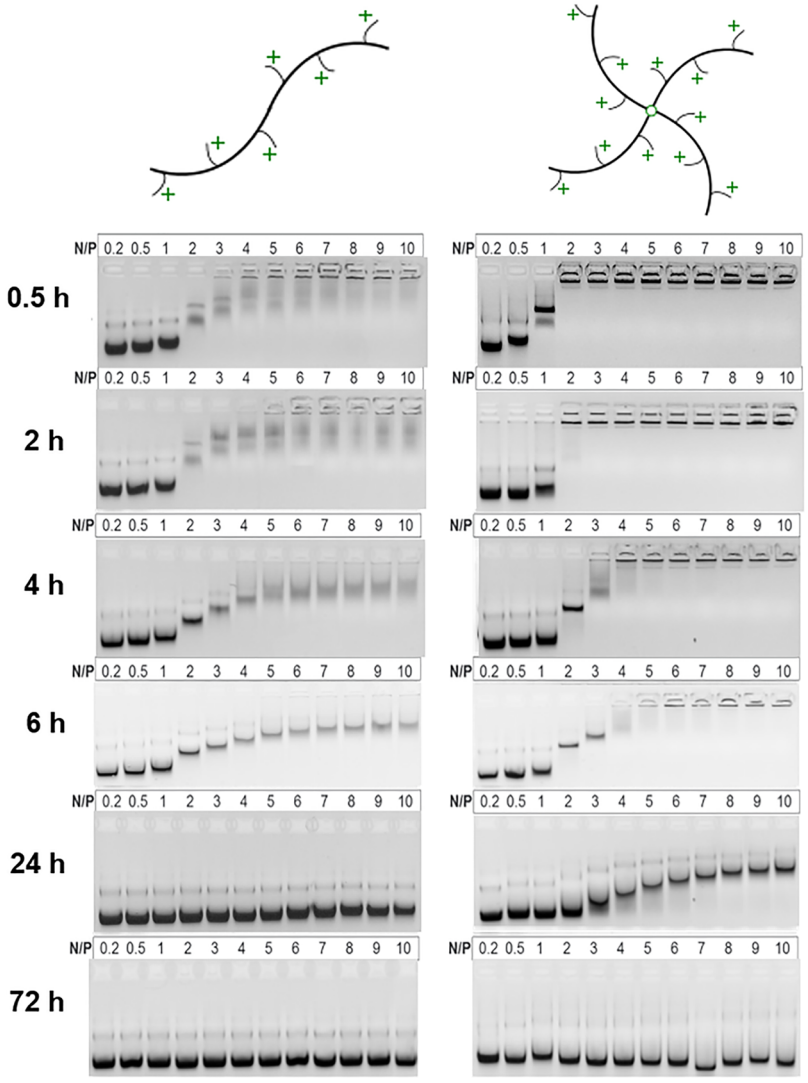

Figure 2. Gel retardation assay with dsRNA and linear/star PDMAEA. Polymer/dsRNA complexes were formed in RNase free water at increasing N^+/P^- ratio (0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) and evaluated after 0.5, 2, 4, 6, 24, and 72 h. Polymer/dsRNA ratios are expressed as molar ratio between polymer ammonium (N+) cationic repeating units and the anionic phosphate groups (P^-) on dsRNA. Samples were incubated at room temperature and loaded onto a 2% w/v agarose gel (100 V, 30 min).

The controlled polymerization of DMAEA is reported to be challenging by all reversible deactivation radical polymerization (RDRP) methods reported to date, mainly due to the high reactivity of the tertiary amine functionality that leads to a large extent of termination and side reactions.[35] In order to circumvent this, the polymerizations of DMAEA are usually stopped at low conversions (~30%) followed by purification and storage of the materials in IPA prior to further use. Under previously reported conditions, the Cu(0)-mediated RDRP[36] of both linear and star PDMAEA were attempted aiming for molecular weights in the range of 5000−6000 g mol^{-1}.[37,38] It is noted that high molecular weight analogues were not targeted as low molecular weight polymers (<10000 g mol^{-1}) have been widely reported to exhibit enhanced solubility when complexed to genetic material and possess much lower toxicity.[27] As such, well-defined linear (M_n = 5600 g mol^{-1}, $Đ$ = 1.18) and star PDMAEA (M_n = 6200 g mol^{-1}, $Đ$ = 1.14) were obtained exhibiting good agreement between the theoretical and experimental values and narrow molecular weight distributions (Figures S1−S3 and Table S1). We also aimed to synthesize the polymethacrylate ((poly(2-(dimethylamino)ethyl methacrylate) PDMAEMA) analogue of PDMAEA, as it is a nonhydrolyzable in aqueous solution so could be utilized as a negative control. However, under identical conditions, the synthesis of PDMAEMA was unsuccessful as evident by the significant broadening of the molecular weight distributions (Figure S4 and Table S2). In order to circumvent this, methyl α-phenylacetate (MBPA) and *N,N,N′,N″,N″*-pentamethyldiethylenetriamine (PMDETA) were instead utilized as the initiator and ligand respectively (as opposed to ethyl-α-bromoisobutyrate (EBiB) and tris[2-(dimethylamino)ethyl]-amine (Me_6Tren) that were used for the linear acrylate polymers).[39] With this optimization, well-defined linear PDMAEMA with low dispersity was obtained (M_n = 6000 g mol^{-1}, $Đ$ = 1.06). Importantly, this polymerization reached full monomer conversion (>99% conversion by ^{1}H NMR) without compromising the molecular weight distribution (Figure S5 and Table S3). To the best of our knowledge, this is the first report of controlled polymerization of DMAEMA via Cu(0)-wire RDRP.

All polymers (linear PDMAEA, linear PDMAEMA, star PDMAEA) were subsequently dissolved into aqueous

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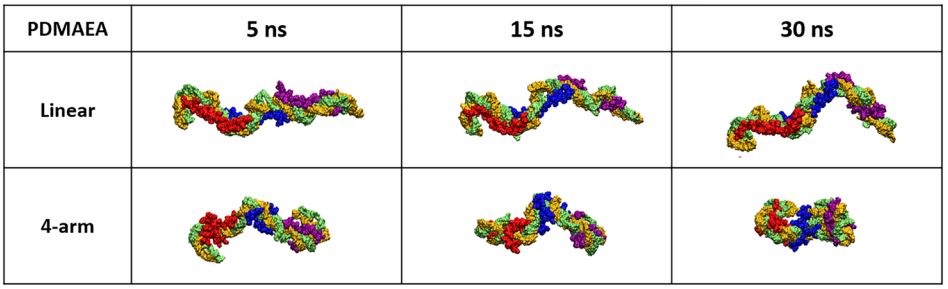

Figure 3. MD simulation snapshots of DNA/PDMAEA complexation for both linear and star polymer during various stages of wrapping process. Simulation shows significant bending of the double helix with the star polymer complex. DNA and polymers are shown in the surface representation in VMD: green and yellow are dsRNA; red, blue, and purple are polymers.

solutions and the extent of the hydrolysis measured via ^{1}H NMR over 50 days.[40] This prolonged time frame is necessary for potential soil applications and previous hydrolysis studies are limited to less than 10 days. The nature of the polymer backbone was initially investigated (methacrylate versus acrylate) with PDMAEMA showing negligible hydrolysis, if any, over the whole-time. (Schemes 1, S1, and S2) This is consistent with previous studies that report the methacrylate analogue to be nonhydrolyzable.[41] In contrast, upon switching from the polymethacrylate to the polyacrylate analogue (linear PDMAEA), hydrolysis occurred rapidly with 11% of the polymer being hydrolyzed within 30 min, followed by a noticeable reduction in the degradation percentage with 25% of hydrolysis in 12 h and 50% in 3 days. The rate of hydrolysis was further decreased reaching 74% over the total period of 50 days. The hydrolysis study was then repeated for the star PDMAEA demonstrating also rapid hydrolysis with a slightly lower degradation percentage than for the linear analogue (Figure 1a and Tables S4 and S5). It is noted that although the architecture seems to have only a small effect on the rate of hydrolysis, the linear polymers reproducibly hydrolyze slightly faster than the star polymers, possibly due to the greater density of cationic nitrogen moieties at the core of the star polymer that are less accessible to water molecules. In order to study the effect of small variations on the molecular weight within these materials, a lower molecular weight star polymer was also synthesized and tested (M_n = 3200 g mol^{-1}, D = 1.12). This experiment revealed a similar rate of hydrolysis when compared to the higher molecular weight star polymer (63% versus 62%). (Figure 1b and Table S6) Thus, changing the molecular weight or the architecture has limited effect on the rate of hydrolysis. This advantage allows the synthesis of PDMAEA with variable molecular weight from batch to batch, while maintaining the reproducible hydrolysis property that is needed for quality control.

The effect of the pH on the hydrolysis was subsequently investigated using star PDMAEA as a model polymer as all polymers, except the PDMAEMA, exhibit comparable hydrolysis rates (Figure 1c and Table S7). The pH of soil can vary significantly depending on the area and the environment with typical values being between 3 and 10.[42] However, in the presence of acid rain and/or inorganic fertilizers even lower pH can be observed in the field.[43] Initially, hydrolysis studies were carried out at natural pH (7.2) and also at pH's 5.5 and 10.1 (the boundaries of physiological pH range). Near identical hydrolysis profiles were observed at all three pHs, demonstrating similar behavior independent of environmental conditions. These results are in agreement with values previously reported for linear PDMAEA.[28,29] With lower pH being common in soil, a further hydrolysis study was carried out at pH ∼ 1.5. Interestingly, a near identical rate of hydrolysis was observed and thus confirming that the rate of hydrolysis is not affected by pH changes (pH 1.5−10.1).

Another factor of importance is the effect of temperature on the degree of hydrolysis, with significant variations within different countries or different seasons. Hence, the hydrolysis at three different temperatures was tested (8, 20, and 37 °C) (Figure 1d and Table S8). The rate of hydrolysis slightly increased upon increasing the temperature, however, the difference in hydrolysis rate between 8 and 37 °C was only 4% at the end of the 50th day, thus showing relatively similar characteristics under significant temperature changes.

To be applicable as a pesticide, dsRNA must bind and subsequently be released into the soil, so it can be absorbed by the plant root. With previous studies limited to the effect of a few polymer properties and environmental factors on the strength of complexation, we further investigated the effect of architecture on the rate of release utilizing gel electrophoresis assays (Figures 2 and S6).[44−47] Polymer/dsRNA complexes (both linear and star) were incubated in RNase free water at increasing N^+/P^- ratios (0.2−10). Then, 0.5 h after incubation, the dsRNA remains loaded within the pockets of the gel (the top band in Figures 2 and S6), indicating strong complexation between all polymers (linear PDMAEA, linear PDMAEMA, star PDMAEA) and dsRNA at an N^+/P^- ratio of 2 or greater. In comparison to the linear polymer, the star PDMAEA illustrated much more complete binding, as shown by dark top band and no smearing in Figure 2 (right). At lower N^+/P^- ratios full binding did not take place as there were insufficient positive charges to bind all of the dsRNA, illustrated by the free dsRNA migrating through the gel. We next examined the ability of these complexes to release dsRNA, as only free dsRNA can be active and most complexes cannot self-release the dsRNA. As expected, the non-hydrolyzable PDMAEMA exhibited no release of dsRNA even after 21 days as no noticeable change in the gel electrophoresis assay was observed (Figure S6).

On the contrary, for linear PDMAEA, smearing could be observed for all N^+/P^- ratios after 30 min (Figure 2 left) suggesting that binding had occurred, and that the partial release had already begun. It is not possible to gain an earlier measurement, as 30 min is the time taken to acquire a gel. This is in contrast to the star PDMAEA, which showed no release until after 4 h for N^+/P^- ratios at 4 or greater. Taken altogether this data demonstrates that the star polymer has a much slower release profile than the linear analogue, with the nearly full release of the dsRNA having occurred for the linear polymer after 24 h, but still some level of binding for the star

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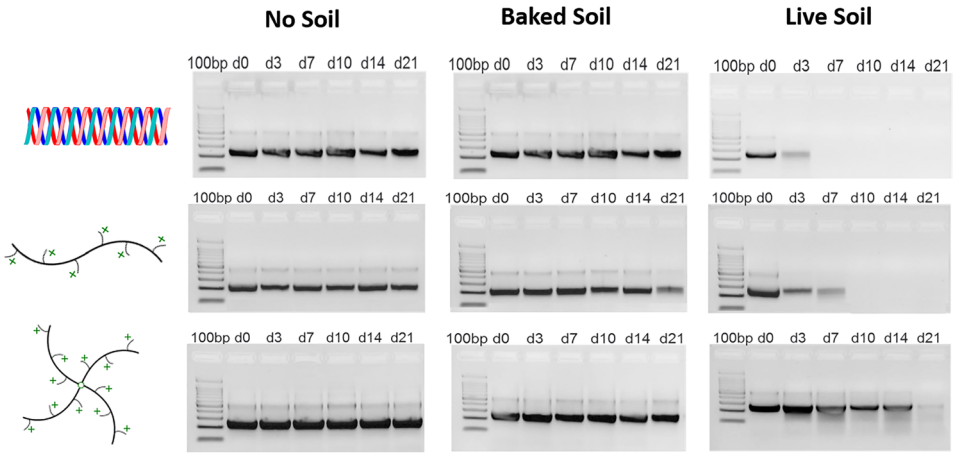

Figure 4. Evaluation of (a) naked dsRNA, (b) linear PDMAEA/dsRNA complex, and (c) star PDMAEA dsRNA complex (200 μL) in no soil, baked soil, and live soil (0.5 g). All polymer/dsRNA complexes were formed incubated at room temperature for different time periods (d = day 0, 3, 7, 10, 14, 21). dsRNA was extracted from soil and samples loaded onto a 2% w/v agarose gel (100 V, 30 min) for subsequent analysis.

polymer was evident. Detailed binding and conformation changes of dsRNA-PDMAEA complexation were subsequently investigated using all-atom molecular dynamics (MD) simulation (Figure 3). Previous reports have shown that the presence of cationic functionalized nanoparticles result in significant bending of DNA.[48] However, there is currently no comprehensive study on the binding of cationic polymers of different architecture to dsDNA. In our simulation, both linear and star PDMAEA (DP40) are strongly bound to the dsDNA and had a profound impact on DNA conformation. The star polymer, however, is more effective in bending and wrapping on the dsDNA, thus a more compact DNA/polymers complex is formed in comparison with the linear one. This is consistent with the gel electrophoresis data, as there is a lower surface area that can potentially come into contact with water or RNases, so better protection and slower release are illustrated.

Another important observation from the gel electrophoresis data, was that the N^+/P^- ratio has a significant effect on the rate of release with increasing the amount of polymer resulting in a much slower rate. Full release of dsRNA is a desired attribute for cationic polymers and is illustrated for all N^+/P^- ratios of both the linear and the star PDMAEA. These polymers are therefore potentially good candidates for soil stability studies, so polyplexes formed from both the linear and star cationic polymers (N^+/P^- ratios of 5) were subsequently investigated (Figure 4). Soil stability assays were conducted by adding samples of polyplex to either live soil or baked soil, with samples incubated for up to 21 days. At each selected time point, TRI Reagent (a mixture of phenol and guanidine thiocyanate) was added which inhibits any RNase activity, preventing subsequent degradation of dsRNA. This also facilitates the extraction of dsRNA from the soil and importantly separation from DNA and proteins. On the addition of chloroform followed by centrifugation, three phases are formed: an aqueous phase containing RNA, an interphase containing DNA, and an organic phase containing proteins.[49] Subsequent enrichment utilizing a lithium chloride procedure allowed for analysis via gel electrophoresis (Materials and Methods in the Supporting Information).[50] Initially, some important control experiments were conducted. In the absence of soil (RNase free solution) the naked dsRNA, the linear PDMAEA/dsRNA complex and the star PDMAEA/dsRNA complex showed no degradation of dsRNA (Figure 4). This is to be expected as in the absence of soil there are no bacteria or enzymes to facilitate degradation. In contrast, when the naked dsRNA was tested in live soil, the intensity of the dsRNA band was greatly reduced after 3 days (Figure 4), suggesting severe degradation of dsRNA. Conversely, when the linear PDMAEA was complexed to the dsRNA in the live soil, distinct bands could be observed after 3 and 7 days, thus clearly showing that complexation to linear PDMAEA was delaying the degradation of dsRNA in soil for around 4 days. However, after 10 days, the dsRNA was completely degraded. Remarkably, when the star PDMAEA was complexed to the dsRNA in live soil the degradation was further delayed with a strong band being observed even after 14days while a much weaker band could still be observed even after 21 days. A further control experiment was undertaken, with the naked dsRNA, the linear PDMAEA/dsRNA complex and the star PDMAEA/dsRNA complex being tested in prebaked soil (240 °C). Having stopped the activity of bacteria and enzymes, no degradation took place confirming that indeed these organisms/enzymes are the only factor responsible for the live soil degradation. As such, it can be concluded that both linear and star PDMAEA can efficiently protect from dsRNA from degradation and extend the lifetime, but importantly the star/dsRNA complex exhibits significantly longer protection when compared to the linear analogue.

In summary, we have identified PDMAEA as a successful polymer for the effective binding and self-release of dsRNA in soil. Both linear and star PDMAEA were successfully synthesized via Cu(0)-RDRP, and the hydrolysis profile of these materials was demonstrated to be independent of environmental conditions thus highlighting the robustness of these candidates. Interestingly the architecture was shown to have a significant effect on binding and release, with the star showing a much slower release rate in comparison to the linear polymer. When applied to soil, star PDMAEA protected dsRNA, illustrating a significantly greater stabilization time of 3 weeks compared to naked dsRNA, which degraded after 3 days. The enhanced stability of dsRNA in soil by complexation to these polymers, followed by a unique self-release mechanism creates many new opportunities for using RNA interference in agrochemical applications.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.8b00420.

Further detailed experimental sectionincluding general procedures for all syntheses and supporting figures (PDF)

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Notes

The authors declare no competing financial interest.

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