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Synthesis of a Diapocynin Prodrug for Its Prolonged Release from Zwitterionic Biodegradable Nanoparticles

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Today, neurodegenerative diseases are affecting more and more individuals. A typical therapeutic target to treat the progression of the symptoms related to these diseases is the proinflammatory microglia. Diapocynin, the dimeric version of apocynin, is often the drug of choice. However, suitable carriers for its controlled delivery are currently lacking in the clinics. In fact, its high hydrophilicity hampers the development of a formulation enabling its sustained release in a biological environment. In this work, the possibility of modifying diapocynin is explored in order to synthesize a prodrug that can be chemically incorporated into biodegradable zwitterionic polymer nanoparticles (NPs). These NPs are synthesized via combination of ring opening polymerization and reversible addition–fragmentation chain transfer (RAFT) polymerization, allowing the incorporation of the desired number of diapocynin units into the carrier and thus to set the drug dosage a priori. The chemical binding of diapocynin avoids its premature release compared to its physical loading inside similar nanovectors. With this strategy, sustained diapocynin release can be achieved for more than 9 days, thus paving the way to a therapy with low number of repeated administrations.

1. Introduction

Neurodegenerative disorders are nowadays affecting an increasing number of people, mainly because of the increase in the average age of the population.[1] Among these diseases, Alzheimer's and Parkinson's diseases, as well as amyotrophic lateral sclerosis (ALS) are the most recognized. ALS patients show progressive weakening and atrophy of muscular tissue as the upper and lower motor neurons of the cerebral cortex and spinal cord continue to degenerate.[2] Almost 90% of ALS cases are sporadic (SALS), while the remaining 10% are family-related (FALS).[3,4] Among the latter, the Cu/Zn-superoxide dismutase mutation (SOD1)[5–7] is responsible for 20% of the cases. The SOD1 mutations were shown to facilitate the development of reactive oxygen species (ROS).[3] Excessive ROS development is associated with Ca^{2+}-dependent enzyme activation including proteases, phospholipases, nucleases, and signaling pathway alterations that lead to mitochondrial dysfunction and neuronal apoptosis.[8,9] However, the exact sequence of time-space events leading to the insurgence and progression of ALS is still debated. This uncertainty determines the lack of successful therapies to stop the gradual loss of neurological functions. Only pharmacological treatments aimed at delaying their course have been proposed so far.[10]

In the studies aimed at developing such treatments, activated microglia-induced neuroinflammation has been recognized as a distinctive characteristic of ALS and as one of the main causes for the development of ALS-related symptoms. In particular, ALS is associated to a strong microglia cells activation and proliferation, with a consequent loss of motor neurons.[11–13] In fact, microglia is an immune cell type located in the central nervous system and is the principal neuroinflammatory mediator.[14] These cells are in a resting state in healthy adults, whereas if neuronal damage occurs, they rapidly cause the release of cytotoxic and inflammatory substances, like oxygen radicals, nitric oxide, and cytokines, which influence surrounding neurons and astrocytes.[14–16] For these reasons, a common therapeutic target for neurodegenerative disorders is the systemic proinflammatory microglia. In this direction, several studies using antibiotics, anti-inflammatory drugs and phenolic antioxidants have been conducted on mice affected by SOD1 mutation in order to improve their life period. Diapocynin, the dimeric form of apocynin, was identified as a promising drug with a therapeutic effect against the progression of the disease. In fact, it was reported that diapocynin can reduce membrane translocation of cytosolic NADPH oxidase

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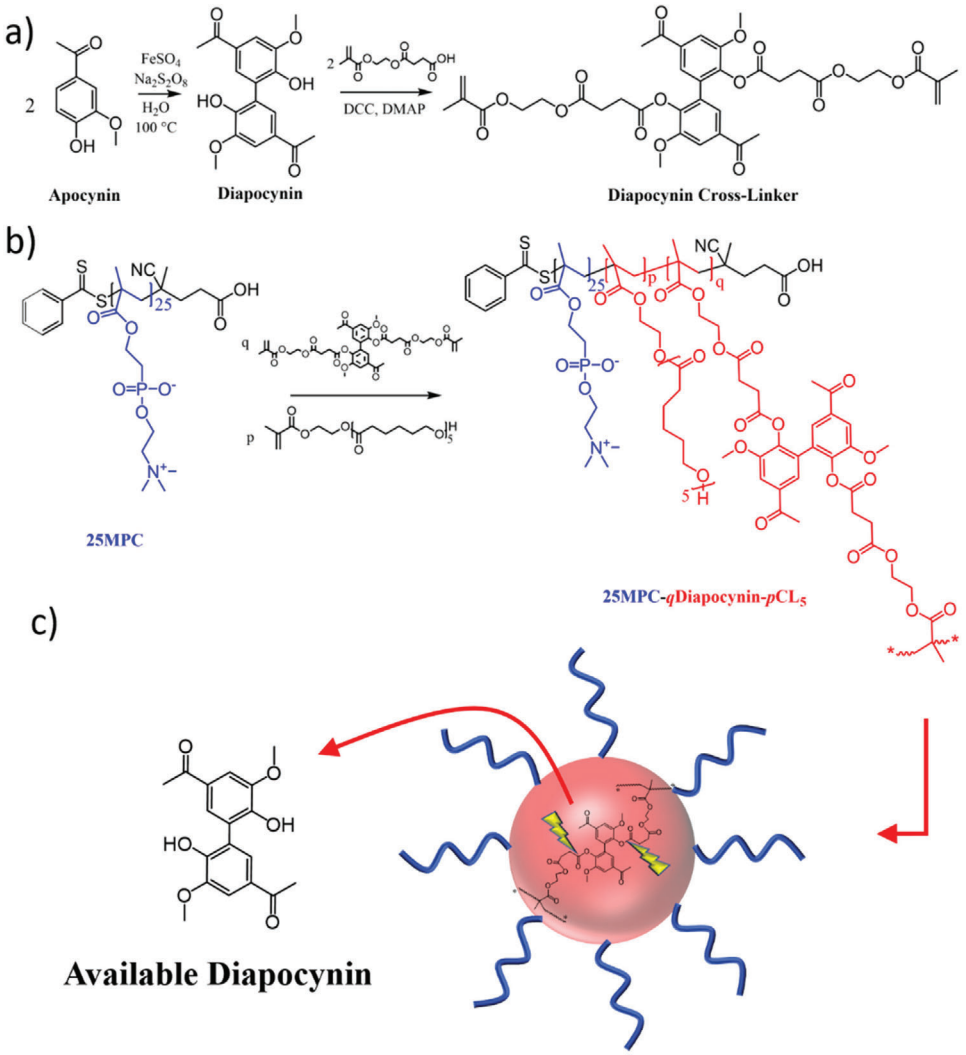
subunits, leading to NADPH oxidase inactivation slowing down the loss of memory and brain damages.[17,18] To access these beneficial effects and then the mitigation of ALS-related symptoms, the prolonged release of diapocynin is crucial. However, diapocynin is currently administered in saline solutions,[18] which leads to short in vivo circulation and rapid clearance through the biological fluids, culminating in poor efficacy of the drug.

The concept of controlled drug delivery, aiming at mediating the release of a drug through a carrier in order to improve its bioavailability and accumulation to the target site may overcome this limitation in the use of diapocynin for the treatment of ALS and in turn increase its therapeutic index. Among all the possible drug carriers investigated so far, polymeric nanoparticles (NPs) are the most attractive.[19,20] In particular, the advent of controlled and living polymerization techniques paved the way to the possibility of designing biodegradable carriers able to guarantee a sustained release for a drug physically encapsulated in these carriers, maintaining its concentration within the desired therapeutic window for long time, and at the same time the complete elimination of the nanovectors after the release.[21–23] In fact, through the combination of controlled radical polymerization and ring opening polymerization (ROP)[24] it is now possible to highly control both the structure and properties of the NPs, which leads to a fine modulation of the drug release rate.[24–27] Moreover, the possibility of controlling the degradation time[28] and the NP size[29] permit to adapt the NP properties to the application. Although appealing, this polymer nanocarriers are suitable for formulating poorly water-soluble drugs. On the other hand, diapocynin is relatively hydrophilic, with a solubility of 42 μg mL^{-1} at pH 7.5 and of 240 at pH 8.5, as this molecule shows a pKa of 7.4 and can be in its anionic form in physiological conditions.[30] Therefore, its encapsulation in polymer NPs would lead to a fast release, within the first hours after the administration, thus preventing a prolonged therapeutic effect.

In this work, we explored the possibility of modifying diapocynin in order to synthesize a prodrug that could be chemically incorporated into degradable zwitterionic polymer NPs. This would prevent the premature diapocynin elimination through the biological fluids, ensuring at the same time a prolonged release and hence reducing the number of required administrations. More specifically, diapocynin was synthesized from apocynin and functionalized with two methacrylate groups by esterification with mono-2-methacryloyloxy succinate (HEMA-succinate). This made the conjugation product amenable for radical chemistry (**Scheme 1**a), thus allowing its chemical incorporation into polymer NPs. At the same time, the ester bonds linking diapocynin to the polymer structure can undergo hydrolysis in aqueous environments and then ensure the possibility of releasing the pure diapocynin when the formulation is administered.

As a proof of concept, the produced diapocynin cross-linker was bound to polymer NPs that are structurally composed of amphiphilic block copolymers produced by reversible addition–fragmentation chain transfer (RAFT) polymerization. The hydrophilic block of the copolymers was synthesized from the zwitterionic phosphorylcholine methacrylate (MPC) and is expected to constitute the shell of the NPs formed in aqueous environments, providing colloidal stability. On the other hand, the hydrophobic, core-forming block is mainly produced from biodegradable macromonomers synthesized via ring opening polymerization (ROP). In particular, by initiating the ROP of ϵ-caprolactone with 2-hydroxyethyl methacrylate (HEMA), it is possible to obtain oligoesters functionalized with a methacrylate group that can be employed for the chain extension of the poly(MPC) macromolecular chain transfer agent (macro CTA) via RAFT emulsion polymerization. The cross-linking and incorporation of diapocynin is achieved at this stage by adding the prodrug to the formulation undergoing RAFT emulsion polymerization (Scheme 1b). In this way, it was possible to covalently bind the prodrug to the NP core making the drug release degradation-dependent (Scheme 1c).[31–33] In fact, we expect the release of diapocynin as soon as the ester bonds anchoring the drug to the polymer network are hydrolyzed in water. Finally, the degradation of the nanovectors leaves the water-soluble poly(MPC)-*b*-poly(HEMA) backbone, with low molecular weight (i.e., <15 000 g mol^{-1}), that can be easily excreted through the biological fluids, thus ensuring no polymer accumulation in the body. After the characterization of these drug-containing nanocarriers, we verified the possibility of achieving a sustained drug release and we studied the influence of the pH on the hydrolytic behavior of the formulation. These results were compared with the release rate of diapocynin either delivered in a saline solution, as the current standard for its administration, or physically entrapped in similar nanovectors. With these studies we confirmed that only by chemically incorporating the diapocynin in the polymer network, it is possible to sustain the drug release for more than 9 days, thus demonstrating the possibility of a prolonged therapeutic effect.

It is worth precising that this work is intended as a preliminary proof of concept of a sustained diapocynin release from polymer nanocarriers, maintaining the drug concentration within a hypothetic therapeutic window. The formulation proposed herein should be carefully evaluated in terms of biocompatibility and biodistribution through suitable in vitro and in vivo tests before claiming it could be exploited in the treatment of neurodegenerative diseases. In particular, the localization of nanovectors in the brain after systemic administration is notoriously a challenge.[34] This is mainly due to the poor penetration of drugs and drug delivery vehicles through the blood brain barrier (BBB), the system of neuronal as well as endothelial cells regulating the molecular transport into the nervous system at the level of the brain-blood interface.[34–36] However, the recent advances in nanomedicine highlighted the critical attributes, in terms of particle size and surface chemistry, allowing to a drug carrier to interact with the endothelial cells at the BBB and to increase the drug concentration in the brain parenchyma.[37–39] Following these discoveries, we were able to accumulate polymer NPs similar to those reported in this study in the nervous system.[40] The NP size played a crucial role in this effective transport together with the transient disruption of the BBB with mannitol. We are therefore confident that following the same strategy, diapocynin could be effectively delivered to the brain. Of course, biodistribution studies in reliable in vivo models become an essential tool for the validation of this work hypothesis and for demonstrating the efficacy of the formulation developed in this work.



Scheme 1. a) Synthesis of the diapocynin cross-linker involving the preliminary dimerization of apocynin to diapocynin and the subsequent DCC-mediated esterification with HEMA succinate. b) Synthesis of cross-linked amphiphilic block copolymers self-assembled into NPs via RAFT emulsion polymerization. c) Schematic representation of the cross-linked NPs and the degradation-driven drug release.

2. Results and Discussion

2.1. Synthesis and Characterization of the Diapocynin Cross-Linker

Diapocynin is the covalent dimer of the NOX2-inhibitor apocynin, an active metabolite formed by myeloperoxidase-mediated oxidation that can also inhibit the expression of the release of NOX2 mRNA cytokines. Diapocynin has a pKa of 7.4, but about half of the hydroxyl groups are deprotonated at physiological pH, thereby allowing the compound to have an overall negative charge that increases its hydrophilicity. Diapocynin was synthesized from apocynin activated with ferrous sulfate and sodium persulfate. The unreacted apocynin was removed by washing three times with boiling water. The proper obtainment of the product with high purity was verified via ESI-MS (**Figure 1**a) and further confirmed by ^{1}H NMR, as shown in Figure 1b.

In past works, we have shown the possibility of loading different lipophilic drugs into polymer NPs through nanoprecipitation, achieving high drug loading efficiencies as well as the control over the drug release during time.[21–23,41,42] This procedure, even though efficient in the formulation of lipophilic drugs, fails with more hydrophilic ones, mainly due to their solubility in water and decreased affinity for the lipophilic environment of the NP core, which prevent to achieve a sustained drug release. Therefore, we decided to chemically incorporate diapocynin in the polymer structure, in order to slow down its release in aqueous environments, making it degradation dependent. In particular, we explored the possibility of synthesizing a diapocynin cross-linker that could be covalently bound to the polymer NPs by reaction with HEMA succinate via DCC-mediated esterification. The possibility of exploiting the reactive –OH groups of diapocynin for functionalization without affecting its therapeutic effect was already shown in the literature.[43] Then, we provided diapocynin with two methacrylate groups that take part in the subsequent

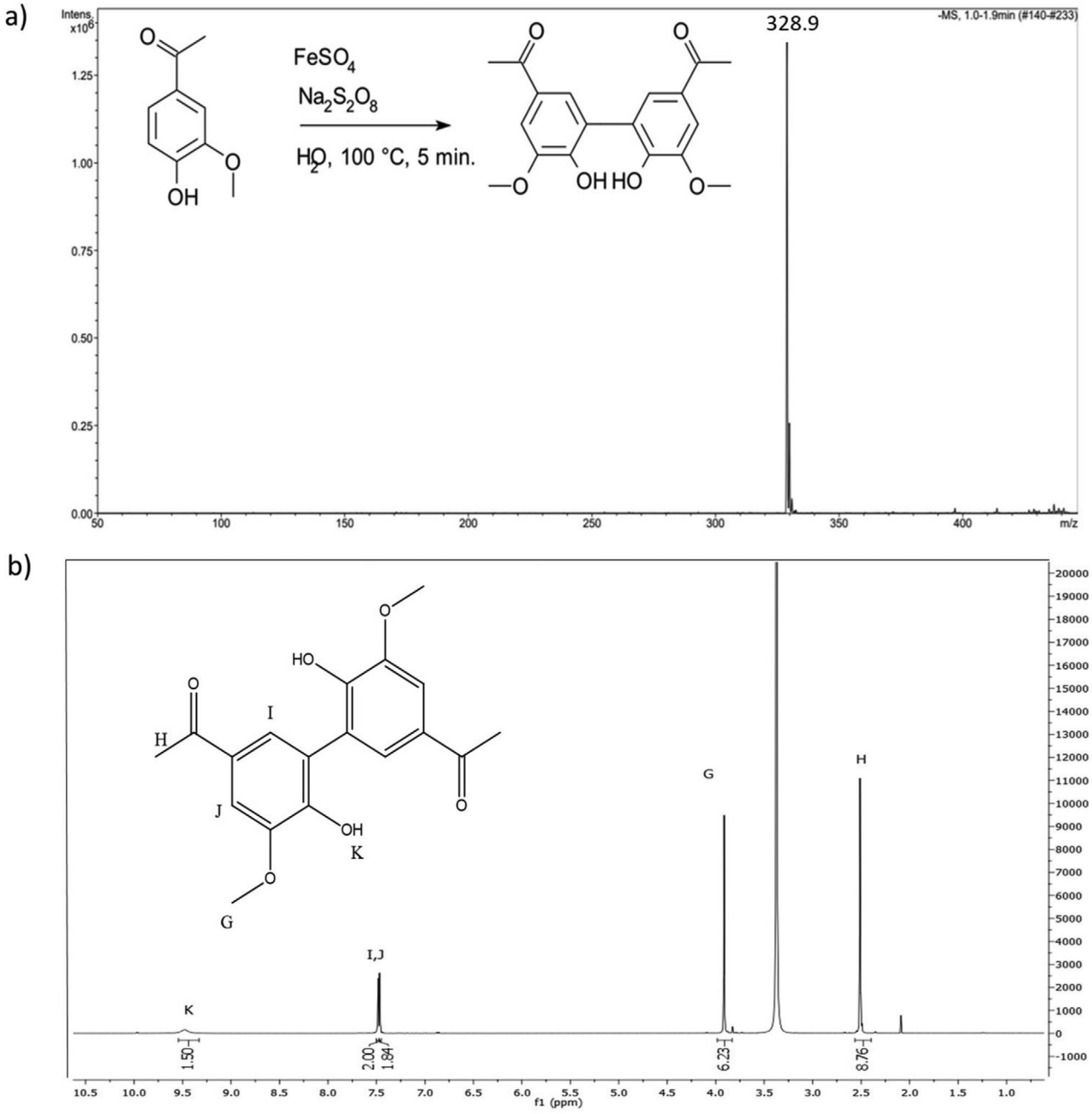


Figure 1. a) Mass spectrum of diapocynin synthesized from apocynin. b) ^{1}H NMR spectrum of diapocynin with peak recognition.

RAFT emulsion polymerization leading to drug-loaded chemically cross-linked NPs. As schematically shown in the structure reported in **Figure 2**a, following this strategy the ester bonds holding diapocynin in the NP core can undergo degradation via hydrolysis during time. This mechanism can then be exploited to induce the sustained release of the drug.

The produced cross-linker was characterized via ^{1}H NMR and mass spectroscopy, as shown in Figure 2a,b, respectively. It is possible to see the high conversion of diapocynin, with no residual free drug after the synthesis. In addition, only a minor percentage (<10%) of monomethacrylate product (m/z = 565 in the ESI-MS spectrum) could be detected, thus testifying the abundance of the desired cross-linker (m/z = 777). Both the species however can be used as prodrugs in the successive polymerization and therefore no further purification was performed.

2.2. Synthesis of Diapocynin-Based NPs and Drug Release

The diapocynin cross-linker, together with HEMA-CL_5, were used to chain extend the 25MPC macro CTA via RAFT emulsion polymerization, leading to chemically cross-linked NPs in

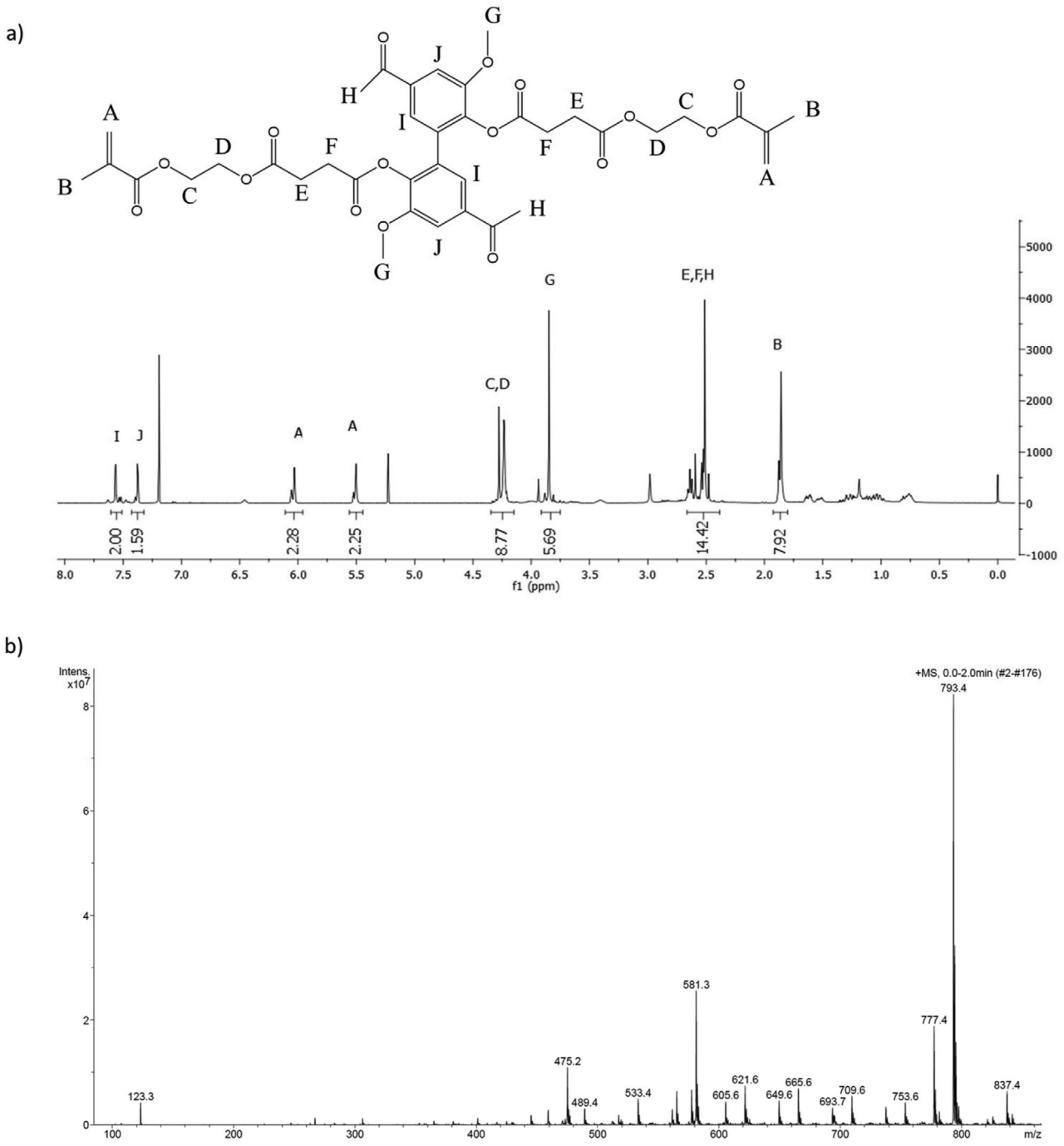


Figure 2. a) ^{1}H NMR spectrum with peak recognition obtained at 400 MHz with 64 scans of the diapocynin cross-linker. b) ESI-MS of the diapocynin cross-linker.

concomitance with the synthesis of block copolymers (for the nomenclature of the different compounds, refer to the Experimental Section). The RAFT polymerization was the technique of choice for the good control it provides over the polymer microstructure, as well as for the possibility of minimizing interchain composition drift, which is essential in this work to ensure that each NP contains a similar and predetermined amount of drug.[44] Different degrees of polymerization of HEMA-CL_5 (p) and diapocynin cross-linker (q) were targeted, in order to find the optimal compromise between NP size, which is expected to increase with p,[28] and the encapsulated drug, increasing with q, without inducing the destabilization of the formulation. It is possible to see in **Table 1** that increasing q at constant p (entries 1–3) led to a first shrinking of the NPs, consequence of the higher cross-linking density. This shrinking is then followed, for more than 2 units of cross-linker, by an increasing size, due to a

Table 1. Nanoparticle characterization in terms of targeted p and q, solid content, volume-average NP diameter (D_v) and polydispersity (PdI).

Entry	*p*	*q*	Solid Content [% w/w]	D_v[nm]	PdI[–]
1	30	1	5%	96.35	0.054
2	30	2	5%	76.45	0.332
3	30	5	5%	309.07	0.418
4	60	1	5%	135.77	0.059
5	15	1	5%	73.61	0.332
6	15	2	5%	78.69	0.279

possible aggregation of NPs that lose their stability. This hypothesis is supported by the increasing PdI, suggesting the formation of polydisperse colloids. Therefore, the maximum number of cross-linker units ensuring narrowly dispersed NPs with D_v suitable for systemic administration was equal to 1. This parameter led to stable NPs also when *p* was systematically varied (entries 1, 4 and 5 in Table 1, see Figure S4 (Supporting Information) in the supporting information). The effect of this variation is the progressive increase in the NP size, in accordance with Equation (1).[28]

$$D = \frac{6 \cdot \mathrm{p} \cdot MW_{HEMA-CL_5}}{N_{Avo} \cdot A_{Cov} \cdot \rho_{b\_lipo}} \quad (1)$$

where N_{Avo} is the Avogadro number, A_{cov} the surface area that a single hydrophilic chain can cover, ρ_{b_lipo} the density of a single lipophilic block, and $MW_{HEMA\text{-}CL5}$ is the molecular weight of HEMA-CL_5.

The formulation leading to the most stable and narrowly dispersed NP suspension was that obtained from $q = 1$ and $p = 30$ (entry 1 in Table 1). The narrow size distribution for these NPs is also confirmed by the TEM micrograph reported in Figure S5 in the Supporting Information, which also testifies the spherical morphology of these colloids.

This NP suspension was then used to study the release rate of diapocynin from the carrier as shown in **Figure 3**. First, we analyzed the release of the drug dissolved in a saline solution to reproduce the current standard for diapocynin administration. This was compared with the release of the drug physically loaded in non-cross-linked zwitterionic NPs (25MPC-30CL_5, i.e., $q = 0$ and $p = 30$). In both cases (Figure 3a), diapocynin was released very fast, mainly due to its hydrophilic nature and poor affinity with the polyester-based NP core. In particular, more than 60% of the drug was released already after 250 min, making these delivery strategies inappropriate for a prolonged drug release. This evidence confirms the necessity of an alternative loading strategy to access a sustained diapocynin release, in order to make it appealing for the treatment of neurodegenerative disorders.

Therefore, we monitored the release of diapocynin from the NPs cross-linked with the diapocynin prodrug. A first comparison with respect to the physical encapsulation can be made based on the amount of diapocynin loaded in the polymer nanovector. With the physical loading strategy described in the Experimental Section, a loading efficiency (i.e., amount of encapsulated drug over the amount of drug added to the formulation) of 79% was

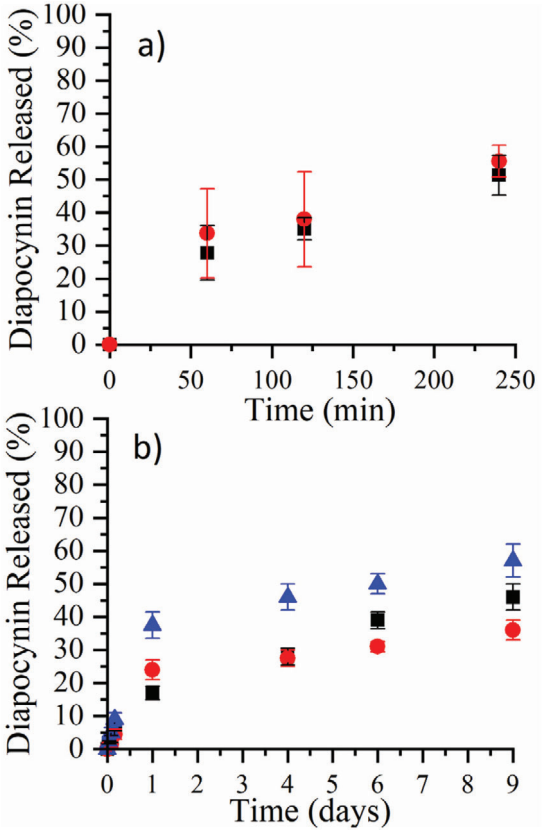


Figure 3. a) Drug release of diapocynin from a saline solution (red circles) and diapocynin physically loaded inside 25MPC-30CL_5 NPs (black squares). b) Amount of released diapocynin from 25MPC-1diapocyinin-30CL_5 NPs (entry 1 in Table 1) at different pH: 3 (black squares), 7 (red circles), 10 (blue triangles).

achieved. This led to a diapocynin loading of 39 mg $g^{-1}_{Polymer}$ without incurring in the suspension destabilization. It is still worth noticing that 60% of the loaded drug is lost already after 4 h. On the other hand, with the formulation 25MPC-1diapocynin-30CL_5 (i.e., $q = 1$, $p = 30$, entry 1 in Table 1) the diapocynin loading was limited to 11 mg $g^{-1}_{Polymer}$ in order to have stable and narrowly dispersed NPs. Nevertheless, this formulation provided significant advantages in terms of release rate. The study of this process was conducted at three different values of pH, namely 3, 7 and 10 and the corresponding amount of released drug during time is reported in Figure 3b. Although the extremes are far from the physiological pH, the investigation at these conditions is important to relate the diapocynin release to the actual degradation of the polymer NPs and to exclude eventual effects of pH on the solubility of the drug.

It can be observed that, in the case of chemically incorporated diapocynin, the release could be extended to up to 9 days. This paves the way to a prolonged drug release and potentially to the achievement of a long-lasting therapeutic window, which enables to reduce the number of administrations, with improved comfort and compliance for the patient. The formulation then showed a pH-dependent behavior and, in particular, a slower release in a neutral environment and a faster release under acidic and alkaline conditions. To prove that this could be due to the catalytic

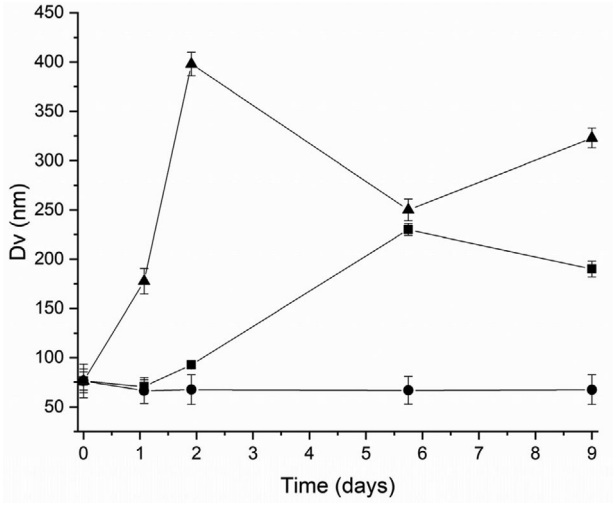


Figure 4. Variation of the NP size during time at pH 3 (squares), pH 7 (circles) and pH 10 (triangles).

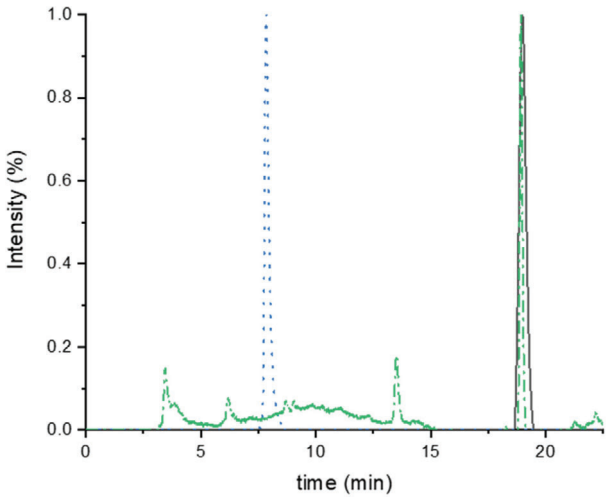


Figure 5. HPLC chromatograms of a diapocynin standard (black full line), diapocynin cross-linker (blue dotted line), and diapocynin released from the polymer NPs (green dash-dotted line).

action of pH on the degradation of the ester bonds linking diapocynin to the polymer network, the data on the drug release were supported by a DLS study[28] aiming at highlighting the NP hydrolytic degradation, as shown in **Figure 4**. In fact, the ester bonds forming the NP core can be hydrolyzed in water, thus causing an initial NP swelling, followed by their progressive dissolution. Therefore, we monitored the NP degradation by tracking their average size during time. In particular, the early increase in the NP diameter measured at pH 10 testified the rapid hydrolysis of the ester bonds inside the NPs and then confirmed the experienced faster drug release measured for the NPs in alkaline conditions than for the NPs in an acidic environment. On the other hand, at pH = 7, the NP size remained stable for 9 days, proving that the integrity of the NPs was preserved for the time of the analysis. This supported the little amount of diapocynin released, as shown in Figure 3b.

Finally, the inspection to the HPLC chromatograms (**Figure 5**) of the released drug confirmed that pure diapocynin could be released from the NPs. The elution peak of the sample collected for the evaluation of the drug release is indeed very similar in terms of shape and retention time to that of the diapocynin standard. This ensures the preservation of the therapeutic effect of the drug after being released from the polymer carrier and hence that the strategy proposed in this work can be effectively adopted for the sustained release of diapocynin. Other species in the chromatogram of the sample hydrloyzed from the NPs between 5 and 15 minutes were actually observed. These peaks were attributed to the oligoesters released together with the drug, which are biocompatible and biodegradable.

3. Conclusions

In this work, we explored the possibility of reaching a sustained release of diapocynin from polymer NPs. Since diapocynin is finding great attention in the treatment of ALS symptoms, this opportunity would increase the therapeutic index of this molecule and improve the patient compliance, thus widening its use in the clinic. However, diapocynin shows a pKa of 7.4 and a water solubility as high as 42 μg mL^{-1} at pH 7.5. This leads to a fast release when the drug is just physically encapsulated in polymer NPs. Therefore, with the aim of slowing down the release of diapocynin for a prolonged delivery, we synthesized a prodrug enabling its chemical incorporation in the polymer chains. The diapocynin cross-linker was produced via DCC-mediated esterification with HEMA succinate and used to synthesize chemically cross-linked NPs via RAFT emulsion polymerization. The number of diapocynin units (q) and hydrophobic units (p) per polymer chain was optimized in order to obtain narrowly dispersed NPs with the highest amount of drug guaranteeing the stability of the formulation. Then, we studied the release of diapocynin from these nanovectors, occurring through hydrolysis of the ester bonds. Despite allowing a lower drug loading compared to the physical encapsulation, with these carriers we were able to prolong the drug release to more than 9 days, with the amount of released diapocynin that is closely dependent on the environment pH. It is worth noticing that the degradation product is pure diapocynin, as confirmed through HPLC. Therefore, the strategy proposed in our work could be successfully employed for increasing the therapeutic index of diapocynin by maintaining its concentration within a hypothetic therapeutic window for prolonged time.

4. Experimental Section

Materials: Apocynin (acetovallinone, 98%, MW = 166.17 g mol^{-1}), deionized water, iron(II) sulfate heptahydrate (99%, MW = 278.01 g mol^{-1}), sodium persulfate(98%, MW = 238.10 g mol^{-1}), sodium hydroxide(98%, MW = 40 g mol^{-1}), hydrogen chloride (37%, MW = 36.46 g mol^{-1}), ϵ-caprolactone (CL, 97% MW = 114.14 g mol^{-1}), stannous octoate (Sn(Oct)2, MW = 405.12 g mol^{-1}), 2-hydroxyethyl methacrylate (HEMA, 97%, MW = 130.14 g mol^{-1}), sodium sulfate (99%, MW = 142.04 g mol^{-1}, ACROS), 4-cyano-4-(phenylcarbonothioylthio)-pentanoic acid (CPA 97%, MW = 279.38 g mol^{-1}), 4,4'azobis (cyanovaleric acid) (ACVA, 98%, MW = 280.2 g mol^{-1}), 2-methacryloyloxyethyl phosphorylcholine (MPC, 97%, MW = 295.27 g mol^{-1}), ethanol (MW = 46.07 g mol^{-1}, Fisher chemical), tetrahydrofuran (THF, MW = 72.11 g

mol^{-1}, Fisher chemical), mono-2-methacryloyloxy succinate (HEMA succinate, MW = 230.21 g mol^{-1}), 4-dimethylaminopyridine (DMAP, MW = 122.17 g mol^{-1}), *N*,*N*'-dicyclohexylcarbodiimide (DCC, MW = 206.33 g mol^{-1}), dichloromethane (DCM, MW = 84.93 g mol^{-1}), dimethyl sulfoxide (DMSO, MW = 78.13 g mol^{-1}), poly(ethylene glycol)methyl ether methacrylate (EG4MA, MW = 300 g mol^{-1}), polyethylene glycol (PEG, MW = 4000 g mol^{-1}). All the solvents and chemicals were purchased from Sigma-Aldrich and used as received unless specifically noted.

Synthesis and Characterization of Diapocynin: Diapocynin was synthetized by activation of apocynin with ferrous sulfate and sodium persulfate following a previously published paper (Scheme 1a).[45] Briefly, 2 g of apocynin were dissolved in 200 mL of boiling deionized water by stirring. 0.15 g of iron(II) sulfate heptahydrate and 1.6 g of sodium peroxydisulfate were added and the stirring continued. Gradually, a brown precipitate formed. After 5 min, the mixture was cooled to room temperature and filtered using a Büchner funnel. The precipitate collected was dissolved in 25 mL of NaOH (3 M) and then re-precipitated by gradually adding 15 mL of HCl (6 M) under strong stirring. The precipitate was filtered and washed with 100 mL of boiling water three times. The product was dried under vacuum overnight. The product was characterized via electrospray ionization-mass spectroscopy (ESI-MS). The mass spectrum was obtained using a Bruker Esquire 3000 PLUS (Esi Ion Trap LC/MSn System) solubilizing 5 mg of sample in 5 mg of methanol. A proton nuclear magnetic resonance (^{1}H NMR) spectrum of diapocynin in deuterated dimethyl sulfoxide (DMSO-d6) was obtained using a Bruker AV 400 (400 MHz) spectrometer, using 64 scans per sample.

Synthesis of Diapocynin Cross-Linker: The prodrug was synthesized from diapocynin and HEMA succinate via DCC-mediated esterification in anhydrous DCM using DMAP as catalyst and DCC as coupling agent (Scheme 1a). More specifically, 0.8 g (2.4 mmol) of diapocynin, 1.394 g (6 mmol) of HEMA succinate and 0.059 g (0.5 mmol) of DMAP were solubilized in 10 mL of anhydrous DCM. The mixture was kept in an ice bath to avoid side reactions. 0.990 g (4.8 mmol) of DCC in 3 mL of anhydrous DCM were added dropwise through a syringe pump (NE-300, New Era Pump Systems) over 1 h, while the mixture was kept under stirring. Finally, the mixture was left heating spontaneously to room temperature, with the reaction proceeding for 24 h. After 24 h, the precipitated dicyclohexylurea was filtered off and the functionalized cross-linker solution was washed twice with distilled water and hydrochloric acid (0.5 M). The solvent was removed under reduced pressure and the obtained material was characterized via ^{1}H NMR in deuterated chloroform ($CDCl_3$) and via ESI-MS as reported before. The product was finally stored at 4 °C.

Synthesis of the Biodegradable Macromonomer: A caprolactone-based macromonomer with five repeating units (hereinafter HEMA-CL_5) was synthesized via ROP of ϵ-caprolactone in bulk conditions, using $Sn(Oct)_2$ as catalyst, and HEMA as chain initiator according to a previously published protocol.[46] Briefly, 3 g (23.07 mmol) of HEMA were mixed with 47 mg (0.116 mmol, i.e., 1/200 eq with respect to HEMA) of $Sn(Oct)_2$ and injected in a mixture of 13.16 g (115.297 mmol, i.e., 5 eq compared to HEMA) of ϵ-caprolactone and 13 mg (0.0925 mmol) of anhydrous sodium sulfate preheated at 125 °C ± 1 °C under magnetic stirring. The reaction was quenched by cooling after 3 h and the obtained material was characterized via ^{1}H NMR in $CDCl_3$ (Figure S1, Supporting Information). The final product was stored at 4 °C and then used without further purification.

Synthesis of the Hydrophilic Macro CTA: A zwitterionic macro CTA (25MPC) was synthesized via RAFT solution polymerization of MPC using CPA as RAFT agent and ACVA as initiator. A degree of polymerization of 25 was targeted by adjusting the monomer to CPA molar ratio to this value, while the ACVA to CPA molar ratio was set to 1/3. Briefly, 1.5 g of MPC (5.08 mmol), 57 mg of CPA (0.2 mmol), and 19 mg of ACVA (0.068 mmol) were dissolved with a final concentration of 20% w/w in a mixture 50/50 v/v of ethanol and acetic buffer (pH = 4.5). The solution was poured in a round bottom flask and purged with nitrogen for 20 min. The mixture was heated to 65 °C and left for 24 h under continuous stirring. The product was then recovered by double precipitation in acetone, filtration and drying at 35 °C under vacuum, and finally stored at −20 °C. Prior to purification, an aliquot of the reaction mixture was collected and analyzed via ^{1}H NMR in deuterated methanol (MeOD-d4) to calculate monomer conversion (X) and degree of polymerization (DP_{MPC}). The NMR spectrum with peak assignment is shown in Figure S2 in the Supporting Information. The molecular weight distribution was analyzed via gel permeation chromatography (GPC) using a Jasco 2000 series chromatograph. The separation was conducted in 0.05 M Na_2SO_4/acetonitrile (80/20 v/v), at a flow rate of 0.5 mL min^{-1} at 35 °C with three Suprema columns (particle size 10 µm and pore sizes 100, 1000, and 3000 Å, Polymer Standards Service). The sample was prepared at a concentration of 4 mg mL^{-1} and filtered through a 0.45 µm nylon filter before injection. All reported values were based on differential refractive index data and relative to polyethylene glycol standards.

NP Synthesis: The NPs were synthesized via RAFT emulsion polymerization of the oligoester-based macromonomer and the diapocynin cross-linker using 25MPC as macro CTA and ACVA as initiator with a total latex concentration equal to 5% w/w. The ACVA to 25MPC molar ratio was kept constant to 1/3 while three diapocynin cross-linker to 25MPC mole ratios (q), namely 1, 2, and 5, and three HEMA-CL_5 to 25MPC mole ratios (p), namely 15, 30, and 60, were investigated (Scheme 1b). As an example, for the NPs composed of the block-copolymer 25MPC-1diapocynin-30CL_5 (q = 1, p = 30), 0.2 g of 25MPC, 0.548 g of HEMA-CL_5, 9.8 mg of diapocynin cross-linker and 2.4 mg of ACVA were dissolved in 21.5 g of 3 × 10^{-3} M acetic buffer (pH = 4.5) and 7.94 g of ethanol. The mixture was purged with nitrogen for 30 minutes and heated in an oil bath at 65 °C under stirring for 24 h. The nanoparticle suspension was dialyzed for 2 days against distilled water, using a regenerated cellulose membrane with a 3.5 kDa molecular weight cut-off (Spectra/Por). The volume-average NP diameter (D_v) and polydispersity index (PdI), were then evaluated via dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS. The samples were diluted in deionized water to a final latex concentration of 0.5% w/w. The signal was recorded at 173° (backscattering). All the reported values are an average of three independent measurements. Transmission electron microscopy (TEM) was performed on a Philips CM200 electron microscope operated at 200 kV and endowed with a Field Emission Gun filament. The NPs were dropped onto a 200-mesh carbon-coated copper grid and dried before analysis. A Gatan US 1000 CCD camera was used to record the images, with a resolution of 2048 × 2048 pixels and 256 grey levels.

To evaluate the differences between the chemical incorporation of the drug inside the NP and its physical loading, we synthesized NPs composed by similar copolymers but without the diapocynin cross-linker, hereinafter 25MPC-30CL_5 (i.e., q = 0 and p = 30), following the same procedure described above. Then, the physical encapsulation of the drug in the preformed NPs was obtained following a method already described in literature.[47] Briefly, 1.5 mg of diapocynin were dissolved in 200 µL of DMSO. The solution was withdrawn with a syringe pre-filled with 3 mL of 25MPC-30CL_5 suspension at 10 mg mL^{-1}. The mixture was ejected and withdrawn three times and finally filtered with a 0.45 µm nylon syringe filter (Sartorius Stedim) to remove the non-encapsulated drug aggregates.

Evaluation of Diapocynin Release via HPLC-UV: Once purified, the drug-loaded NP suspensions 25MPC-1diapocynin-30CL5 and 25MPC-30CL_5 were poured in dialysis cassettes (Spectra/Por, molecular weight cut-off 3.5 kDa) and dialyzed against 10 × 10^{-3} M phosphate buffers at three different pH, i.e., 3, 7, and 10, to evaluate the drug release rate. In particular, the different pH values were chosen to investigate the role of this parameter on the hydrolytic release of diapocynin from the prodrug. An additional sample constituted by the diapocynin dissolved in physiological solution (NaCl 9 g L^{-1}) at 2 mg mL^{-1} was added to a separate cassette and dialyzed against a 10 × 10^{-3} M phosphate buffer at pH 7 to evaluate the release rate of the free drug (i.e., without any form of polymer drug delivery system). The release profile was investigated by taking aliquots of 1 mL of the dialysis buffers outside the cassettes at predetermined time points (1, 2, 4, 6, and 9 days for 25MPC-1diapocynin-30CL_5 and 60, 120, and 240 min for the free drug and 25MPC-30CL_5 with diapocynin physically entrapped). The solutions were freeze-dried using a Telstar-Lyoquest freeze-drier working at 0.1 mbar and −56 °C and the solid residue dissolved in 1 mL of acetonitrile for evaluation of the diapocynin content via high performance liquid chromatography (HPLC). An Agilent 1100 series chromatograph equipped with a diode array detector was used. Reverse-phase HPLC was performed on a C18 analytical column (250 mm × 4.6 mm, particle size 5 µm), recording the signal at 276 nm. A gradient elution from

0.1% v/v phosphoric acid aqueous solution to 60:40 v/v acetonitrile/0.1% phosphoric acid solution in 30 minutes was performed. The eluent composition was kept constant for 3 minutes and then brought back to 100% phosphoric acid solution in 10 minutes. The injection volume was 20 µL. For quantitative analysis, diapocynin standards (1–80 µg mL^{-1}) were prepared in acetonitrile. Data were fit to a straight line by linear regression analysis (Figure S3, Supporting Information).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

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