Poly (β-amino ester)-co-poly(caprolactone) terpolymers as non-viral vectors for mRNA delivery in vitro and in vivo

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The production of new proteins with messenger RNA (mRNA) has gained a broad interest due to its potential for addressing a wide range of diseases. Here we report on the design and characterization of novel ionizable poly (β-amino ester)-co-poly(caprolactone) terpolymers, synthesized via the combination of the ring opening polymerization and the Michael step-growth polymerization. The versatility of this method has been demonstrated by varying the number of caprolactone units attached to each PBAE terpolymer. The ability of the novel PCL-based PBAE materials to deliver mRNA has been shown to depend on the physiochemical characteristics of the material, such as lipophilicity, as well as the formulation method used to complex the polymer with the oligonucleotide. This latter variable represents a previously unstudied aspect of PBAE library screens that could play an important role in identifying true top candidates for nucleic acid delivery. The most stable terpolymer was injected via IV in mice and showed a transfection efficacy several times higher than the PEI which was focused in the spleen, opening the possibility to use these biodegradable carriers in the intravenous delivery of antigen-encoding mRNA for cancer immunotherapy and vaccination.

The intracellular delivery of exogenous oligonucleotides has the potential to treat a wide range of diseases ranging from cancer to genetic disorders[1]. The production of new proteins with messenger RNA (mRNA) has recently garnered particular interest, given that it need only to be delivered to the cytoplasm (where the cellular translation machinery is located) and does not pose the risk of the genomic integration associated with DNA[2]. For this reason,

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mRNA has been investigated as a strategy for protein replacement therapy and has shown potential in several fields, including cancer immunotherapy[3, 4], apoptosis-associated diseases[4, 5], vaccines[6] and genomic engineering[7], amongst others.

However, efficacious delivery of naked mRNA to the site of interest has proven challenging, owing in part to its clearance by the immune system, its inability to cross the cell membrane and potential degradation in lysosomes[2]. For this reason, several carriers, such as lipids, polymers and hydrogels, have been designed to enhance mRNA delivery and, thus, the protein expression and duration[8]. Cationic polymers are among the materials that have been studied for gene delivery given their ability to electrostatically bind and condense nucleic acids to form nanoparticles, such as polylysine[9], polyethylenimine (PEI)[10], chitosan[11], and poly(dimethyl aminoethyl methacrylate)[12]. Poly (β-amino ester)s (PBAEs) are particularly promising due to their facile synthesis, transfection efficiency and degradability[13, 14]. Such polymers have already shown potential to deliver DNA in a variety of *in vitro*[15, 16, 17] and *in vivo*[18] contexts. PBAEs for gene delivery are often produced in two steps that consist in (i) a step growth polymerization (Michael addition) between amines and a diacrylate[13] and (ii) an end-capping of the final polymer with a diamine[19]. Depending on their chemistry, the multiple ester bonds present in the polymer backbone may be degraded in the body via esterases and hydrolysis[20]. One possible degradation product are diols which are derived from the spacer of the diacrylate that it is used in the first step of the PBAE synthesis[17] (**Figure S1** in the Supporting Information). The effect of these degradation products in the context of *in vivo* nanoparticle delivery of nucleic acids is difficult to determine a priori, but we reasoned that designing degradation products with known safety profiles could further ensure biocompatibility of these materials.

Poly-caprolactone (PCL) and poly-lactic acid (PLA) based polymers are commonly used biodegradable polymers in the biomedical field due to their biodegradability and biocompatibility[21]. However, they do not possess positive charges, and are therefore difficult to directly complex with anionic nucleic acids for gene delivery[22]. To overcome this issue, PCL and PLA are typically combined with cationic polymers. For example, Arote et al synthesized a PCL-based diacrylate and used it to synthesize a branched poly(ester amine) by reacting with a low molecular weight polyethyleneimine (PEI)[23]. These polymers were reported to successfully complex DNA into NPs with reduced toxicity and higher transfection efficiency compared to the ones made of PEI 25 K[23]. Interestingly, complete hydrolysis of this material should result in hydroxycaproic acid and ethylene glycol, as well low molecular

weight PEI[23]. The diacrylates in their study were synthesized by acylation of one commercially available and low molecular weight (M_n= 530) PCL-based diol, limiting synthetic flexibility. The aim of this work is to substitute the currently adopted diacrylates in the synthesis of PBAE terpolymers with PCL-based derivatives that are expected to be more biocompatible.

Towards this end, PCL-based diacrylates were synthesized via a two-step procedure that consists of (i) a ring opening polymerization (ROP) of caprolactone (CL) with hydroxyethylacrylate (HEA) as initiator and tin(II)-ethylhexanoate as catalyst and (ii) acylation with acryloyl chloride (**Figure 1a**). The ROP is a so called "living polymerization", a technique that allows for control over the number of the units of caprolactone (q) that can be incorporated into the final diacrylate. In addition to PCL having known biocompatibility[24], the tunability of q allows for control over the diacrylate's (and therefore the PBAE's) lipophilicity and molecular weight by varying the reaction stoichiometry. In addition, the polymerization does not require any solvent or further purification steps due to the nearly complete conversion and the absence of by-products[25]. For this reason, the obtained macro-monomer can be esterified with acryloyl chloride without the need for additional purification. This acrylation step was used to attach the additional double bond required to produce the PBAEs via Michael step-growth polymerization. All of these polymeric diacrylates can be seen as derivatives of ethylene glycol diacrylate (**Figure 1b**, compound **A**), which has a q value equal to 0 and has been used in previous PBAEs[17]. Three different PCL-based diacrylates were synthesized with a low dispersity ($Đ$ < 1.3) and theoretical q values very close to the one evaluated via ^{1}H-NMR (data in **Table S1** and representative ^{1}H-NMR of a custom diacrylate in **Figure S2** in the Supporting Information). PBAEs incorporating the custom diacrylates were synthesized in a two-step reaction modified from a previous protocol[17]. In the first step, an alkyl amine (dodecyl amine) and different hydrophilic amines (**1**, **2**, **3**, **4**) were reacted with the custom diacrylates (**A, B, C, D**) at a ratio equal to 1.2:0.7:0.3 (diacrylate: hydrophilic amine: dodecyl amine) to produce a library of acrylate-terminated PBAE terpolymers (**Figure 1b**). The choice to incorporate a lipophilic amine into the PBAE is justified by the superior stability and efficiency of the terpolymers to deliver DNA compared to classic PBAEs, as previously demonstrated[17]. The second step is carried out without any intermediate purification and consists of another Michael addition with an excess of a PEGylated diamine in order to completely consume the

remaining double bonds in the mixture. In fact, the end-capping of the PBAEs has been shown to increase both the transfection efficiency and the tolerability of PBAEs[16, 26].

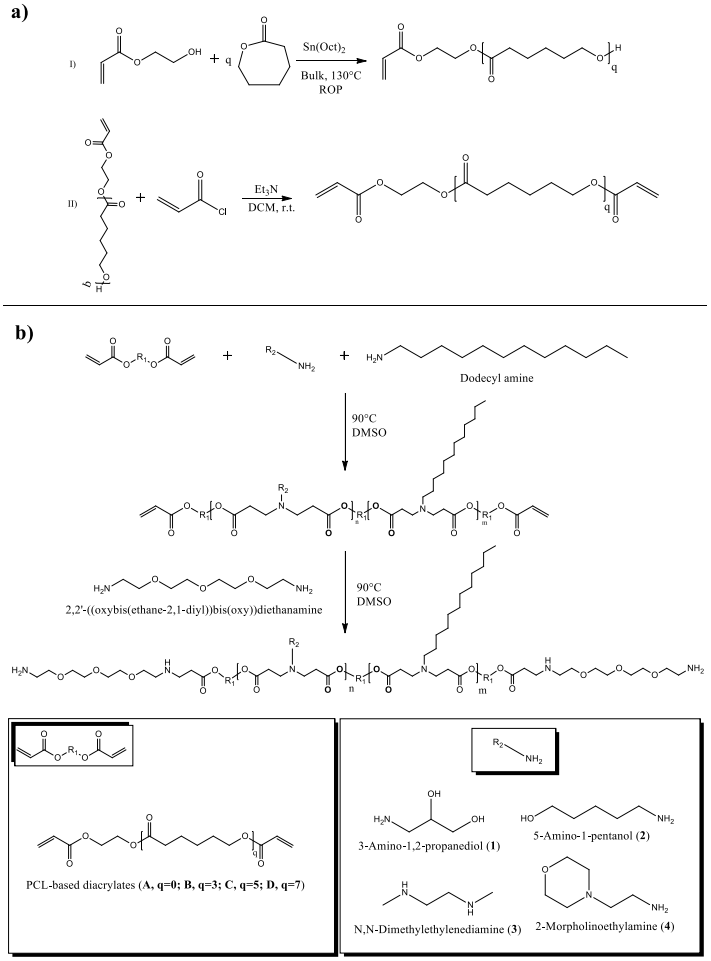

Figure 1 (**a**) Synthesis of the poly-caprolactone-based diacrylates (**b**) Synthesis of PBAE terpolymers

The PBAE terpolymers synthesized show a high *Đ* (**Table S2** in Supporting Information) as expected from a step-growth polymerization (*Đ* ≥ 2)[27, 28]. However, the terpolymers synthesized with the same hydrophilic amine (e.g. A1, B1, C1, and D1) show a linear correlation with Mw, corresponding to the Mw of the diacrylate (**Figure 2a**). In fact, the M_w obtained via GPC is a linear function of the number of CL units attached with the intercept

equal to the M_w of the diacrylate **A** due to the control given by the ring opening polymerization. This strategy allowed us to improve the control over the M_w and the lipophilicity of the final material. However, it is important to note that as long as the number of amines per polymer chain is theoretically the same for a pre-assigned stoichiometry[28], the increase of the M_w results in a reduction by weight of the overall ionizable charges (i.e. charge density).

With the polymers in hand, we next focused our attention on formulating the polymers into mRNA nanoparticles. The lipophilicity of the polymer also plays an important role in the production of the NPs since it can affect its solubility in the medium that is used for the ionic complexation of the oligonucleotides. For this reason, two different procedures were adopted in order to evaluate the effect of the formulation method on the NPs size and, in turn, on their transfection efficiency in vitro. In the first case, so called direct mixing, the polymer solution (polymer in DMSO) was directly dissolved in 25 mM NaOAc and then mixed with the mRNA as also reported in Eltoukhy et al[17]. However, not all the polymers are freely soluble in this aqueous phase and small nanoparticles were produced. The addition of the oligonucleotide led to a partial coagulation of these primary NPs into larger nanoaggregates, as visible in **Figure S3**. In the second case, the polymer solution was dissolved in ethanol before mixing with mRNA. In this case, referred to as pre-mixing protocol, the NPs were formed during the mixing of the two phases resulting into a higher size compared to the ones obtained with the previous method (**Figure S3**). However, as visible in **Figure 2b**, all the NPs generated with the pre-mixing protocol show a higher transfection efficiency compared to the ones obtained via direct mixing. This effect is more enhanced where the lipophilicity of the terpolymers is higher, and underscores the importance of formulation conditions, in addition to material synthesis, when conducting material screens. As a positive control for the in vitro transfection test, we used a top-performing PBAE terpolymers (DD90) identified in a previous study[29]. DD90 has been shown to have a transfection efficacy several times higher than jetPEI (a commercially available PEI transfection reagent) with the direct mixing protocol[29], but when it is formulated with the pre-mixing protocol its efficacy also increases by ~20x, with luciferase expression comparable to the polyester-based terpolymer **C2**. Thus, in order to find the best performing materials in the screening of the PBAE library, the pre-mixing procedure was applied to all the terpolymers, which were then used to transfect HeLa Cells (**Figure 2c**). A dependence on the lipophilicity of the terpolymer on the transfection

efficiency can be seen in all the tested formulations. In particular, the less lipophilic PBAEs that are based only on the **A** diacrylate show little efficacy in mRNA delivery while the initial increase in the number of the CL units (q) leads to a higher transfection. However, as mentioned previously, the beneficial impact of the increased lipophilicity is counterbalanced by the reduction in the density of ionizable amines that are necessary to correctly condense the mRNA. When this reduction is too high, it affects the transfection, as visible in the terpolymers made with **D**. In addition, as visible in the cell viability test in **Figure 2d**, almost all the tested terpolymers were not cytotoxic at the doses tested.

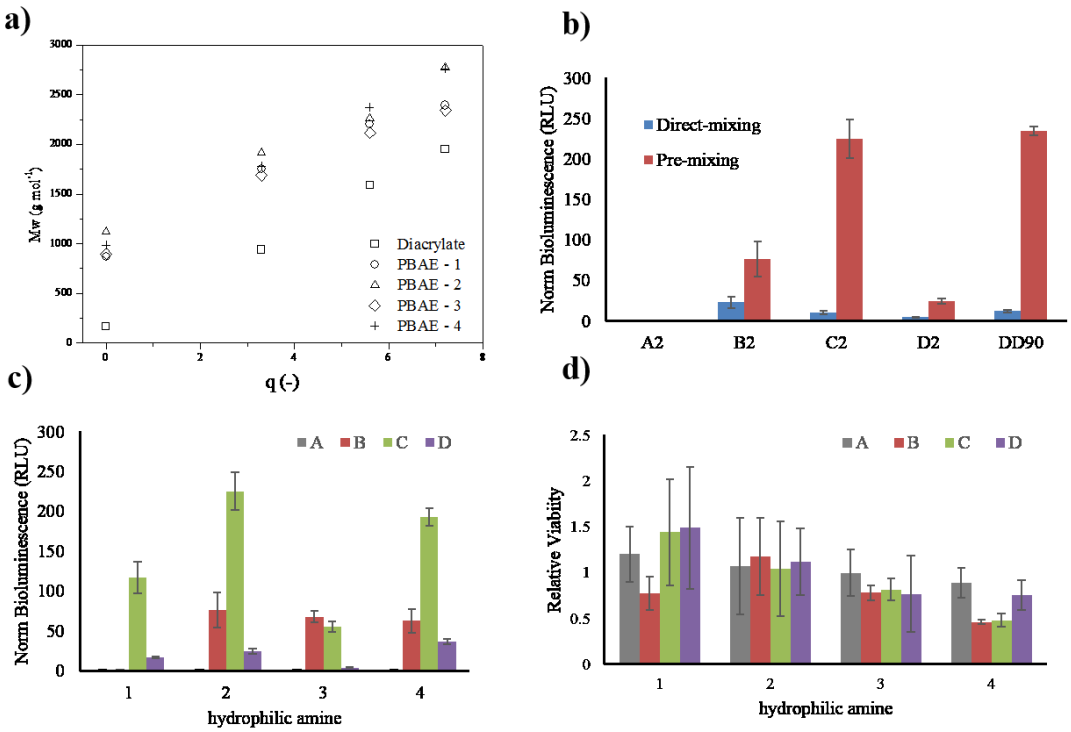

Figure 2 (**a**) Mw of the custom diacrylates and the PBAEs with different hydrophilic amines (**b**) Transfection in HeLA cells of a PBAE series (2) with the two different formulation procedures using 50 ng/well of a firefly luciferase mRNA (complete sequence is reported in the SI). DD90[29] PBAE used as positive control. (**c**) Transfection in HeLA cells with the new procedure for all the synthesized PBAEs using 50 ng/well of a firefly luciferase mRNA (complete sequence is reported in the SI) (**d**) HeLa cell relative viability for all transfection conditions shown in **Figure 2** (**c**) (2.5 µg/well of PBAE). Data are presented as mean ±SD, n=4;

The stability of the NPs in biological fluid is relevant to performance when systemic delivery of the mRNA is required. For this reason, the top performing terpolymers were co-formulated with 7 wt.% of a polyethylene glycol-modified lipid (PEG-lipid) and the stability of the resulting NPs were tested in a mixture of 10% fetal bovine serum (FBS) according to a previous protocol[29]. Briefly, the NPs were incubated at 37°C and the optical clarity of the solution was evaluated at different time points (**Figure 3a**). Among the **C** derived terpolymers, the **C1** showed the best stability and was therefore optimized in order to find the minimum amount of PEG-lipid that led to no change in the absorbance once incubated with the FBS, (**Figure 3b)**. The increase in PEG-lipid led to a higher stability, but at the same time it reduced the transfection efficacy of the NP formulation due to the charge shielding effect of the PEG tails, as already reported in literature[29] and also demonstrated in **Figure S4**.

Previous work has indicated that serum stability is required for effective systemic delivery of PBAEs[29], so **C1** was formulated with 15 wt.% PEG-lipid for *in vivo* testing with an mRNA coding for firefly luciferase. This formulated **C1** was injected in mice at three different doses (0.5, 0.25 and 0.125 mg/kg mRNA) and their efficacy and toxicity compared with **PEI**. Interestingly, this PCL-based terpolymer has showed delivery primarily to the spleen (**Figure 3c**), in contrast with other members of the PBAE family[29] that also show activity into the lungs, as well as lipid nanoparticles that are typically effective in the liver[30]. In addition, even at the highest tested concentration, there is no significant difference in levels of the liver enzymes ALT and AST **(Figure 3d)** or in body weight (**Figure S5**) compared to PBS ($p>0.05$) indicating little systemic toxicity caused by the formulation at the doses tested.

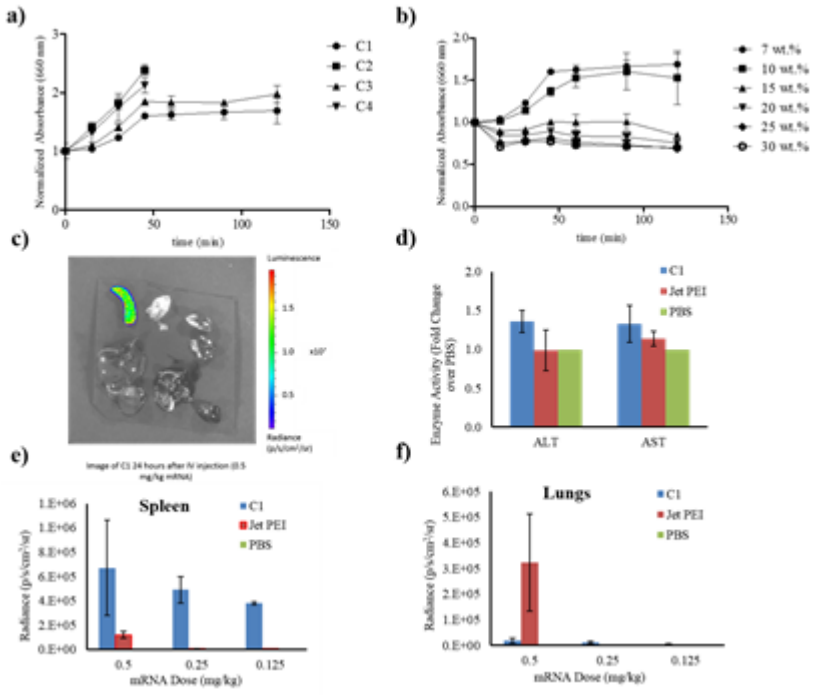

Figure 3: (**a**) Serum stability of PBAE terpolymers with 5 CL unit (C) at 7 wt.% of PEG-Lipid (**b**) Serum stability of **C1** at different wt.% of PEG-Lipid (**c**) Image of **C1** 24 hours after IV injection (0.5 mg/kg mRNA). (**d**) Liver enzime activity of **C1** and Jet PEI at a dose of 0.5 mg/kg (**e**) Average radiance of **C1** and JET PEI in the spleen after 24 hours at different mRNA doses (**f**) Average radiance of **C1** and JET PEI in the lungs after 24 hours at different mRNA doses. Data are presented as mean ±SD, n=3;

Given its tendency to deliver to the spleen, this class of PBAEs may be find utility as a vehicle for the delivery of mRNA to immune cells, as recently shown with lipid formulations[31].

In summary, we have developed a synthesis for the production of poly-caprolactone based diacrylates. The versatility of this method has been demonstrated by varying the number of caprolactone units attached to each diacrylate and is to some extent reflected in the characteristics of the PBAE terpolymers synthesized from them via step-growth polymerization. The ability of the novel PCL-based PBAE materials to deliver mRNA has been shown to depend on the physiochemical characteristics of the material, such as lipophilicity, as well as the formulation method used to complex the polymer with the oligonucleotide. This latter variable represents a previously unstudied aspect of PBAE library screens that could play an important role in identifying true top candidates for nucleic

acid delivery. The most stable terpolymer, the **C1**, was injected via IV in mice and showed a transfection efficacy several times higher than the **PEI** which was focused in the spleen, opening the possibility to use these biodegradable carriers in the intravenously delivery of antigen-encoding mRNA for cancer immunotherapy and vaccination. We envision that the adoption of a controlled living polymerization for the synthesis of more biocompatible and more biodegradable custom diacrylates from other lactones will lead to the synthesis of more versatile poly-beta-amino esters with more controlled properties, higher efficacy and lower toxicity for a variety of applications.

Experimental Section

PCL-based diacrylates with 3,5 and 7 monomer units were synthesized in a two-step procedure: (i) ring opening polymerization of CL initiated by HEA at 130°C with tin(II) 2-ethylhexanoate as catalyst and (ii) direct esterification of the previous product at room temperature with acryloyl chloride. The PBAE terpolymers were synthesized by reacting the diacrylates, amines and alkyl amines in DMSO at a molar ratio of 1.2:0.7:0.3 for 48 h at 90°C. The end-capping of the polymer was performed by adding to the solution the diamine at room temperature and letting to react for an additional day at 90°C. The final concentration of the polymer solutions was adjusted to 100 mg ml^{-1} and then they were stored at -80°C. DD90 was synthesized according to a previously published protocol (DD90-C12-122)[29]. Two different methods were used to produce the NPs. In the direct mixing protocol, 5 µl of the 100 mg ml^{-1} polymer solution in DMSO were dissolved in 195 µl of 25 mM NaOAc and then mixed by pipetting with 200 µl of a solution of 50 ng/µl of mRNA in 25mM NaOAc buffer. In the pre-mixing protocol, 5 µl of the 100 mg ml^{-1} polymer solution in DMSO were dissolved in 45 µl of ethanol with different amount of PEG-lipid when specifically noted and mixed by pipetting with 50 µl of a solution of 200 ng/µl of mRNA in 25mM NaOAc buffer. The final mixture was finally diluted with PBS to reach the mRNA final concentration of 5 ng/µl.

Detailed experimental procedures and characterization methods are reported in the Supporting Information

Supporting Information

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

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References

[1] J. C. Kaczmarek, P. S. Kowalski, D. G. Anderson, Genome medicine **2017**, 9, 60.
[2] O. Sergeeva, V. Koteliansky, T. Zatsepin, Biochemistry (Moscow) **2016**, 81, 709.
[3] E. Gilboa, J. Vieweg, Immunological reviews **2004**, 199, 251.
[4] M. A. McNamara, S. K. Nair, E. K. Holl, Journal of immunology research **2015**, 2015.
[5] A. Matsui, S. Uchida, T. Ishii, K. Itaka, K. Kataoka, Scientific reports **2015**, 5.
[6] R. Weiss, S. Scheiblhofer, E. Roesler, E. Weinberger, J. Thalhamer, Expert review of vaccines **2012**, 11, 55; C. Pollard, S. De Koker, X. Saelens, G. Vanham, J. Grooten, Trends in molecular medicine **2013**, 19, 705.
[7] H. Wang, H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng, F. Zhang, R. Jaenisch, Cell **2013**, 153, 910; M. Hashimoto, T. Takemoto, Scientific reports **2015**, 5; J. L. Gori, P. D. Hsu, M. L. Maeder, S. Shen, G. G. Welstead, D. Bumcrot, Human gene therapy **2015**, 26, 443.
[8] K. J. Kauffman, M. J. Webber, D. G. Anderson, Journal of Controlled Release **2015**.
[9] D. W. Pack, A. S. Hoffman, S. Pun, P. S. Stayton, Nature reviews Drug discovery **2005**, 4, 581.
[10] S. C. De Smedt, J. Demeester, W. E. Hennink, Pharmaceutical research **2000**, 17, 113.
[11] G. Borchard, Advanced drug delivery reviews **2001**, 52, 145.
[12] S. Agarwal, Y. Zhang, S. Maji, A. Greiner, Materials Today **2012**, 15, 388.
[13] D. G. Anderson, D. M. Lynn, R. Langer, Angewandte Chemie **2003**, 115, 3261.
[14] J. C. Sunshine, D. Y. Peng, J. J. Green, Molecular pharmaceutics **2012**, 9, 3375.
[15] D. Zhou, Y. Gao, A. Aied, L. Cutlar, O. Igoucheva, B. Newland, V. Alexeeve, U. Greiser, J. Uitto, W. Wang, Journal of Controlled Release **2016**; S. Y. Tzeng, B. P. Hung, W. L. Grayson, J. J. Green, Biomaterials **2012**, 33, 8142; J. Kim, J. C. Sunshine, J. J. Green, Bioconjugate chemistry **2013**, 25, 43; H. Guerrero-Cázares, S. Y. Tzeng, N. P. Young, A. O. Abutaleb, A. Quiñones-Hinojosa, J. J. Green, ACS nano **2014**, 8, 5141.
[16] J. Sunshine, J. J. Green, K. P. Mahon, F. Yang, A. A. Eltoukhy, D. N. Nguyen, R. Langer, D. G. Anderson, Advanced Materials **2009**, 21, 4947.
[17] A. A. Eltoukhy, D. Chen, C. A. Alabi, R. Langer, D. G. Anderson, Advanced Materials **2013**, 25, 1487.
[18] J. Zhao, P. Huang, Z. Wang, Y. Tan, X. Hou, L. Zhang, C.-Y. He, Z.-Y. Chen, ACS applied materials & interfaces **2016**; J. C. Sunshine, S. B. Sunshine, I. Bhutto, J. T. Handa, J. J. Green, PloS one **2012**, 7, e37543; A. Mangraviti, S. Y. Tzeng, K. L. Kozielski, Y. Wang, Y. Jin, D. Gullotti, M. Pedone, N. Buaron, A. Liu, D. R. Wilson, ACS nano **2015**, 9, 1236; C. D. Kamat, R. B. Shmueli, N. Connis, C. M. Rudin, J. J. Green, C. L. Hann, Molecular cancer therapeutics **2013**, 12, 405.

[19] G. T. Zugates, W. Peng, A. Zumbuehl, S. Jhunjhunwala, Y.-H. Huang, R. Langer, J. A. Sawicki, D. G. Anderson, Molecular Therapy **2007**, 15, 1306; G. T. Zugates, N. C. Tedford, A. Zumbuehl, S. Jhunjhunwala, C. S. Kang, L. G. Griffith, D. A. Lauffenburger, R. Langer, D. G. Anderson, Bioconjugate chemistry **2007**, 18, 1887.
[20] K. A. Whitehead, J. R. Dorkin, A. J. Vegas, P. H. Chang, O. Veiseh, J. Matthews, O. S. Fenton, Y. Zhang, K. T. Olejnik, V. Yesilyurt, Nature communications **2014**, 5, 4277.
[21] A. Kumari, S. K. Yadav, S. C. Yadav, Colloids and Surfaces B: Biointerfaces **2010**, 75, 1; D. Bobo, K. J. Robinson, J. Islam, K. J. Thurecht, S. R. Corrie, Pharmaceutical Research **2016**, 33, 2373.
[22] I. E. Palamà, B. Cortese, S. D'Amone, G. Gigli, Biomaterials science **2015**, 3, 144.
[23] R. Arote, T.-H. Kim, Y.-K. Kim, S.-K. Hwang, H.-L. Jiang, H.-H. Song, J.-W. Nah, M.-H. Cho, C.-S. Cho, Biomaterials **2007**, 28, 735.
[24] M. A. Woodruff, D. W. Hutmacher, Prog. Polym. Sci. **2010**, 35, 1217.
[25] R. Ferrari, Y. Yu, M. Morbidelli, R. A. Hutchinson, D. Moscatelli, Macromolecules **2011**, 44, 9205.
[26] A. A. Eltoukhy, D. J. Siegwart, C. A. Alabi, J. S. Rajan, R. Langer, D. G. Anderson, Biomaterials **2012**, 33, 3594.
[27] W. Cheng, D. Wu, Y. Liu, Biomacromolecules **2016**, 17, 3115.
[28] W. H. Carothers, Transactions of the Faraday Society **1936**, 32, 39.
[29] J. C. Kaczmarek, A. K. Patel, K. J. Kauffman, O. S. Fenton, M. J. Webber, M. W. Heartlein, F. DeRosa, D. G. Anderson, Angewandte Chemie **2016**.
[30] K. J. Kauffman, J. R. Dorkin, J. H. Yang, M. W. Heartlein, F. DeRosa, F. F. Mir, O. S. Fenton, D. G. Anderson, Nano letters **2015**, 15, 7300; O. S. Fenton, K. J. Kauffman, R. L. McClellan, E. A. Appel, J. R. Dorkin, M. W. Tibbitt, M. W. Heartlein, F. DeRosa, R. Langer, D. G. Anderson, Advanced Materials **2016**, 28, 2939.
[31] L. M. Kranz, M. Diken, H. Haas, S. Kreiter, C. Loquai, K. C. Reuter, M. Meng, D. Fritz, F. Vascotto, H. Hefesha, Nature **2016**; O. S. Fenton, K. J. Kauffman, J. C. Kaczmarek, R. L. McClellan, S. Jhunjhunwala, M. W. Tibbitt, M. D. Zeng, E. A. Appel, J. R. Dorkin, F. F. Mir, Advanced Materials **2017**, 29.

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