Chapter 21

Preparation of mRNA Polyplexes with Post-conjugated Endosome-Disruptive Peptides

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Abstract

Successful delivery of mRNA into the cytosol of professional antigen-presenting cells (APCs) poses one of the biggest challenges in developing effective mRNA vaccines to treat various cancers and viral infectious diseases. However, most polymeric mRNA delivery systems fail to transfect APCs. We have discovered that decoration of pH-sensitive endosome-disruptive GALA peptides on the surface of mRNA polyplexes leads to efficient targeting and transfection of APCs. GALA peptides not only enhance specific uptake in APCs through binding to sialic acid moieties, they also facilitate the endosomal escape of mRNA especially in dendritic cells (DCs). Here, we describe in detail the production of stabilized mRNA polyplexes post-conjugated with GALA peptides via copper-free click chemistry. Methods described here include the synthesis and purification of GALA peptides and its conjugation to mRNA polyplexes.

Key words mRNA polyplexes, Peptide conjugation, GALA peptide, Nanoparticle surface decoration, Post-modification, Post-PEGylation, Click chemistry

1 Introduction

mRNA based therapeutics especially mRNA vaccines have recently come into focus as a promising new alternative to conventional vaccine approaches because of their high potency, suitability for rapid development, low manufacturing costs, and safe administration [1]. The success of mRNA vaccine relies on efficient delivery of mRNA into the cytosol of professional antigen-presenting cells (APCs) to express antigens for intracellular processing in the MHC Class I pathway or cytokines for immune modulation. Various strategies have been applied to enhance the functional delivery of mRNA to APCs. One of them is employing pH-sensitive and membrane disruptive peptides such as GALA to facilitate the endosomal escape of mRNA needed for effective translation.

GALA is a viral fusion protein-mimicking 30-mer peptide, with a repeating sequence of glutamic acid–alanine–leucine–alanine,

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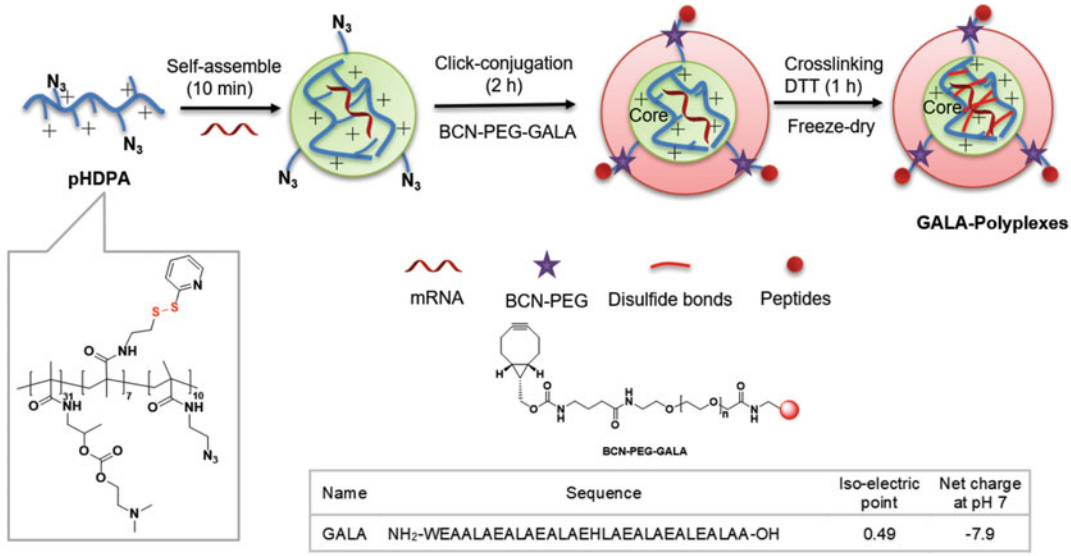

Name	Sequence	Iso-electric point	Net charge at pH 7
GALA	NH_2-WEAALAEALAEALAEHLAEALAEALEALAA-OH	0.49	-7.9

Fig. 1 Schematic illustration of the 3-steps preparation method of GALA modified mRNA polyplexes (reproduced from ref. 5 with permission from ACS Publications)

which can trigger endosomal escape events (Fig. 1) [2]. GALA peptides have been applied to insert on the surface of liposomes to aid cytosolic delivery of DNA and siRNA [3, 4]. Due to its negative charge at neutral pH, the peptide has been scarcely used in cationic polyplex formulations, as the electrostatic attraction can trigger polyplexes aggregation. Recently, we have demonstrated that crosslinked mRNA polyplexes coated with GALA peptides can afford efficient cellular uptake and mRNA transection in DCs (~50%) [5]. Our further mechanistic studies have unveiled the dual function of GALA peptide towards polyplexes uptake and intracellular trafficking in DCs—it both targets sialic acid-terminated glycans on the DC surface and mediates the endosomal escape of mRNA to the cytosol. Previous studies have shown that GALA must be present on the surface of particles to exert its function [6, 7]. To engineer the surface exposure of GALA peptides, we demonstrated that a post-conjugation method can effectively prevent GALA peptides from being embedded into the particle core—the functional shell with GALA was introduced to the pre-formed mRNA polyplexes using copper-free click chemistry (Fig. 1).

The original research article was published in *bioconjugate chemistry* [5], focused mainly on the in vitro evaluation of the GALA modified mRNA polyplexes. In this chapter, we describe an optimized and detailed protocol for the GALA peptide synthesis and post-conjugation to mRNA polyplexes.

2 Materials

All chemical reagents were of the highest available purity (> 98%) and used without further purification. Cationic p(HPMA-$DMAE_{31}$-co-$PDTEMA_7$-co-$AzEMAm_{10}$) (pHDPA) random copolymer and bicyclononyne (BCN)-PEG_{5000}-COOH were synthesized as previously reported [8].

2.1 Reagents

pHDPA polymer p(HPMA-$DMAE_{31}$-co-$PDTEMA_7$-co-$AzEMAm_{10}$) (*see* **Notes 1** and **2**).

Bicyclononyne (BCN)-PEG_{5000}-COOH.

EGFP-mRNA Enhanced green fluorescent protein mRNA (TRiLink biotechnologies, San Diego, CA).

Cy5-EGFP-mRNA (TRiLink biotechnologies, San Diego, CA).

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid).

DCC Dicyclohexylcarbodiimide.

NHS N-hydroxysuccinimide.

NH_4HCO_3 Ammonium bicarbonate.

H-Ala-2-ClTrt resin.

Fmoc-Leu-OH.

Fmoc-Glu(Otbu)-OH.

Fmoc-Ala-OH.

Fmoc-Trp(boc)-OH.

Fmoc-His(trt)-OH.

Oxyma Ethyl cyanohydroxyiminoacetate.

DIC *N,N′*-Diisopropylcarbodiimide.

TIS Triisopropylsilane.

TEA Triethylamine.

TFA Trifluoroacetic acid.

Nuclease-free water.

2.2 Solvent

DCM Dichloromethane.

DMF *N,N′*-Dimethylformamide.

Diethyl ether.

HFIP Hexafluoroisopropanol.

2.3 Buffer Solutions (See *Note 3*)

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a resistivity of 18 MΩ-cm at 25 °C).

1. 20 mM acetate buffer (pH 5): Add 0.57 mL glacial acetic acid to 480 mL water and adjust the pH to 5.0 with 1.0 M and 0.1 M NaOH. Adjust the total volume to 500 mL with water. Opened bottles should be stored in the refrigerator and used within 1 month.
2. 20 mM HEPES buffer (pH 7.4): Add 2.38 g HEPES to 480 mL water and adjust the pH to 7.4 with about 1 mL

4 M NaOH. Adjust the total volume to 500 mL with water. Closed bottles with sterilized solutions can be stored at room temperature and may be used up to 1 year after preparation. Opened bottles should be stored in the refrigerator and used within 2 months.

3. 50% Sucrose/HEPES buffer (pH 7.4): Dissolve 20.0 g sucrose in 30 mL 20 mM HEPES buffer. Add buffer until the volume reaches 40 mL.
4. 5 M sodium chloride: dissolve 2.9 g of NaCl in 8 mL water. Add water until the volume is 10 mL.
5. 50× TAE buffer: Dissolve 242.0 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0) in reversed osmosis water to 1 L. Adjust pH to 8.0. Sterilize the buffers by filtration through filters with 0.22-μm pore sizes and autoclave.
6. 1 M NH_4HCO_3 buffer (pH 8.5): Dissolve 7.9 g in 80 mL water, adjust pH to 8.5. Add water volume to 100 mL.
7. Stock solution of pHDPA (10 mg/mL): Weight 10.0 mg pHDPA polymer in RNase-free microfuge tubes and dissolve in 1 mL of 20 mM acetate buffer. Vortex until totally dissolved and store at −20 °C until used, but within 3 months (*see* **Note 4**).
8. 1,4-Dithiothreitol solution (DTT, 100 mM): Weight and dissolve 7.7 mg DTT in 0.5 mL water in RNase-free microfuge tubes. Vortex until totally dissolved and use within 1 h.

3 Methods

3.1 BCN-PEGylation of GALA Peptide (Fig. 2)

3.1.1 Synthesis of BCN-PEG-NHS

1. Dissolve 55.0 mg (10.5 μmol, 1 equiv) of BCN-PEG-COOH, 6.4 mg (31.4 μmol, 3 equiv) of DCC, and 3.6 mg (31.4 μmol, 3 equiv) of NHS in 1 mL dry DCM.
2. Stir the mixture overnight at room temperature.
3. Filter the reaction mixture with 0.2 μM Phenex syringe filter (PTFE, polytetrafluoroethylene) to remove the formed and insoluble dicyclohexylurea (DCU).
4. Precipitate the filtrate into 50 mL diethyl ether, centrifuge (5000 × *g*) to pellet BCN-PEG-NHS.
5. Re-dissolve BCN-PEG-NHS in 1 mL dry DCM, and precipitate the solution in 50 mL diethyl ether, centrifuge (5000 × *g*) to pellet BCN-PEG-NHS. Repeat this step for one time.
6. Collect the pellet and dry overnight under vacuum to get BCN-PEG-NHS.

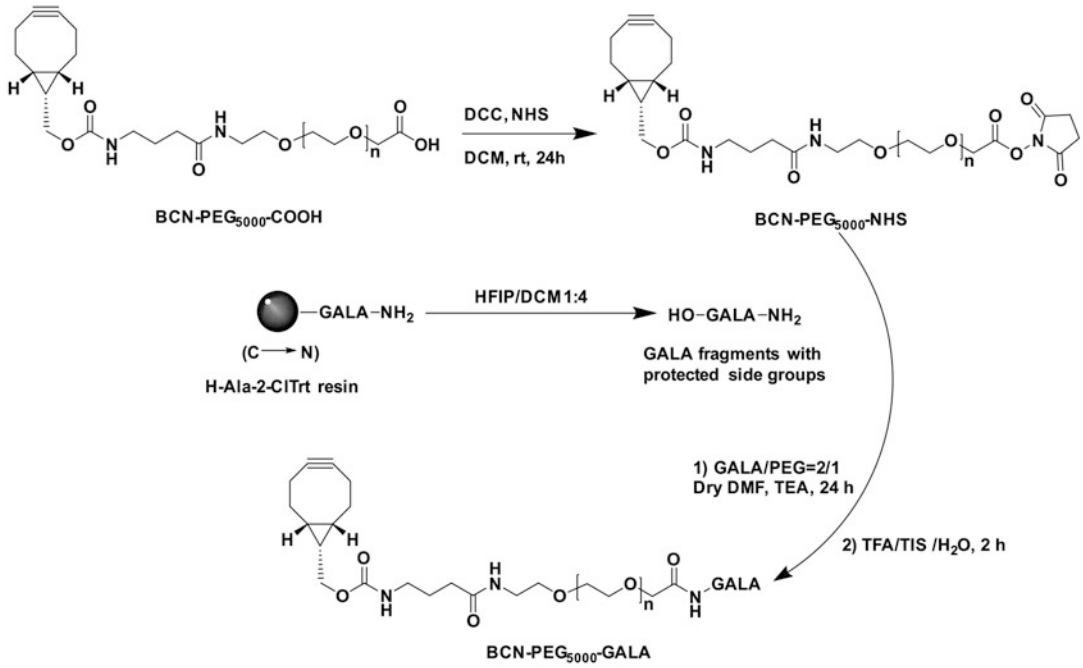

Fig. 2 Synthesis scheme of BCN-PEG-GALA. The clickable BCN-PEG-GALA can be synthesized by coupling (1R,8S,9s)-Bicyclo[6.1.0]non-4-yn-9-ylmethyl N-PEG-succinimidyl carbonate (BCN-PEG-NHS) to the N-terminus of protected GALA fragments (*see* **Note 5**)

3.1.2 Synthesis of Side Chain Protected GALA-Amine Peptide

1. Use an automated microwave-assisted peptide synthesizer (Liberty Blue peptide synthesizer, CEM corporation, Matthews, NC, US) to synthesize the side chain protected GALA sequence.
2. Weigh 200 mg of preloaded H-Ala-2-ClTrt resin (0.1 mmol scale) as the starting reagent for the buildup of the GALA sequence. Each amino acid addition cycle consists of an Fmoc-deprotection step followed by an Fmoc-amino acid coupling step.
3. For the Fmoc-deprotection step, apply 5 mL of 20% piperidine in DMF to the resin for 2 min at 90 °C. Regarding the Fmoc-amino acid coupling step, apply 5 mL of reaction cocktail (0.5 mmol of Oxyma, 0.5 mmol of DIC, 0.5 mmol Fmoc-amino acids in 5 mL DMF) to the Fmoc-deprotected resin for 10 min at 90 °C.
4. After the eventual buildup of GALA-amine sequence, add 3 mL HFIP/DCM (1/4) to the resin for 30 min to cleave the side chain protected GALA-amine peptide off.
5. Use reduced pressure evaporation (Buchi Rotovap R-200, 35 °C) to remove the HFIP/DCM and obtain the side chain protected GALA peptide as white solid (~85% yield), and use thereafter without purification (*see* **Note 6**).

3.1.3 Coupling BCN-PEG-NHS with Side Chain Protected GALA-Amine

1. Dissolve 32.0 mg of BCN-PEG-NHS (5.9 μmol, 1 equiv), 51.0 mg of side chain protected GALA-amine (12.0 μmol, 2 equiv), and 5.0 μL TEA (36.0 μmol, 6 equiv) in 2 mL dry DMF.
2. Stir the reaction mixture overnight at room temperature under argon atmosphere.
3. Precipitate the solution into 100 mL diethyl ether, centrifuge (5000 × *g*) to pellet the PEG-protected GALA, and re-dissolve the pellet in 1 mL dry DMF.
4. Repeat precipitation in diethyl ether twice, and dry the pellet overnight under vacuum.

3.1.4 Deprotection of BCN-PEG-GALA

1. Dissolve BCN-PEG-GALA (side chain protected) in 4 mL of cleavage cocktail (TFA/TIS/H_2O at a molar ratio of 95/2.5/2.5, *see* **Note 7**), and stir for 2 h.
2. Precipitate the deprotected BCN-PEG-GALA in 100 mL diethyl ether, centrifuge (5000 × *g*) to pellet the crude products. Repeat precipitation in diethyl ether for 2 times and dry overnight under vacuum.
3. Dissolve the 30 mg dried pellet in 500 μL of 1 M NH_4HCO_3 and dilute with ultrapure water to 2.5 mL.
4. Load on PD 10 column (pre-equilibrated with 5 × 5 mL of 5 mM NH_4HCO_3) and elute with 3.5 mL of 5 mM NH_4HCO_3, collect the filtrate and freeze-dry (Buchi, Lyovapor L-300, −40 °C, <1 mbar) to get BCN-PEG-GALA (*see* **Note 8**).
5. Dissolve 10.0 mg freeze-dried BCN-PEG-GALA in an RNase-free microfuge tube with 1.0 mL 20 mM HEPES buffer. Vortex until it is totally dissolved and store at −20 °C for use within 3 months.

3.2 mRNA polyplex Preparation

In this step, prepare mRNA polyplexes with different Nitrogen (polymer)/Phosphate (mRNA) (N/P) ratio with a final mRNA concentration of 20 μg/mL for Subheading 3.3 agarose gel assay.

1. Clean the surface of work bench with RNaseZAP™ and 70% ethanol. Use RNase-free filter tips for all the pipetting work.
2. Dilute 2.4 μL of EGFP-mRNA (1 mg/mL) with 57.6 μL of 20 mM HEPES buffer (pH 7.4) to get a mRNA solution of 40 μg/m. Aliquot them into RNase-free PCR tube with 10 μL each.
3. Prepare 100 μL of pHDPA polymer with a concentration of 384 μg/mL (N/P 8, $W_{polymer}/W_{mRNA} = 9.6$), 288 μg/mL (N/P 6), 192 μg/mL (N/P 4), 96 μg/mL (N/P 2), and 48

μg/mL(N/P 1) from stock solution (10 mg/mL) with 20 mM HEPES buffer.

4. Add 10 μL of pHDPA solution to 10 μL of mRNA solution (40 μg/mL), pipetting up and down for 20 times (*see* **Note 9**).
5. Incubate for 10 min at room temperature before usage in Subheading 3.3.

3.3 Gel Retardation Assay to Find Optimal (N/P) Ratio (See Note 10)

1. Prepare 1× TAE buffer in reversed osmosis water by diluting 10 mL of 50× TAE in 490 mL reversed osmosis water.
2. Prepare 1% agarose gel by adding 1 g agarose to 100 mL 1× TAE in a 250 mL conical flask. Heat in a microwave oven until the agarose has completely dissolved.
3. Cool the solution to about 60 °C and add 5 μL GelGreen (Bio-Connect BV, Huissen, the Netherlands) and mix well. Pour the agarose solution into the electrophoresis tray with a comb positioned, check for air bubbles (*see* **Note 11**).
4. After the gel has completely set (30–45 min at room temperature), carefully remove the comb and place the gel tray in the electrophoresis tank. Make sure the loading lane is close to the cathode. Add enough working buffer 1× TAE to completely submerse the gel with about 1–2 mm of TAE buffer on top.
5. Mix 20 μL of the mRNA polyplexes (Subheading 3.2) with 4 μL 6× Loading Dye and load into a slot of the submerged agarose gel.
6. Start electrophoresis at continuous 120 V for 30 min, the mRNA retardation was examined using a Gel Doc XR+ system (BioRad Laboratories Inc., Hercules, CA) with Image Lab software (Fig. 3).

3.4 Preparation of GALA Modified mRNA polyplexes (See Note 12)

In a typical experiment, polyplexes are prepared at a N/P ratio of 4, with a final mRNA concentration of 100 μg/mL (*see* **Note 13**). Carry out all procedures at room temperature unless otherwise specified.

1. To prepare sterile polyplexes, switch on the biohazard or laminar airflow safety cabinet 30 min before use and clean the surface with RNaseZAP™ and 70% ethanol. Use RNase-free filter tips for all the pipetting work.
2. Dilute the 50 μL of mRNA (1 mg/mL) stock solution with 153 μL of HEPES buffer.
3. Add 76 μL of HEPES buffer to 24 μL of pHDPA (10 mg/mL) stock solution. A solution of 240 μg pHDPA in 100 μL buffer is obtained.
4. Add in one injection the 100 μL of diluted pHDPA solution to the 203 μL of diluted mRNA solution. A 303 μL sample

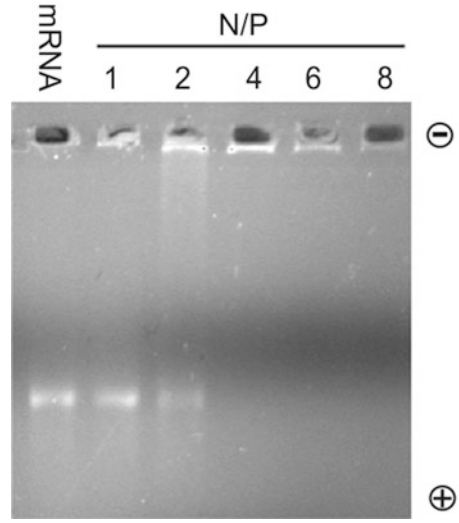

Fig. 3 Agarose gel retardation assay of mRNA polyplexes prepared at different N/P ratios. mRNA was completely bound to pHDPA polymer at N/P ratio 4 or higher

containing 50 μg mRNA and 240 μg pHDPA is obtained (*see* **Note 14**).

5. Immediately, pipet up and down for 20 times and incubate for 10 min at room temperature.
6. Add 104 μL of BCN-PEG-GALA (10 mg/mL) (*see* **Note 15**) to the polyplex mixture and mix well, incubate at room temperature for 2 h (*see* **Note 16**).
7. Add 41.4 μL of dithiothreitol (DTT, 1 mM, dilute 5 μL stock with 495 μL water) to the GALA modified mRNA polyplexes and incubate for 1 h at room temperature to activate crosslinking (*see* **Note 17**).
8. Add 50 μL of 50% sucrose/HEPES buffer and 13 μL of sodium chloride (5 M) to a final 5% sucrose and 150 mM salt concentration. Mix the solution well and snap-freeze in liquid nitrogen and freeze-dry overnight (*see* **Note 18**).
9. Upon use, add 500 μL of RNase-free water to the dried mRNA polyplex sugar cake, let it stand for 30 min, pipetting up and down before adding them to the cells.

4 Notes

1. The polymer used in this protocol consists of three components: carbonic acid 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacryloylamino)-ethyl ester (HPMA-DMAE) [9], N-[2-(2-pyridyldithio)]ethyl methacrylamide (PDTEMA)

[10, 11], and 2-azidoethylmethyacryl amide (AzEMAm) [8], which provide the positive charges needed to complex mRNA, disulfide bridges for self-crosslinking and azides for regioselective post-conjugating of GALA peptides, respectively. The polymer has an average number molecular weight of 11.1 kDa, with a polydispersity of 1.9. The polymer contains 505 nmol/mg pyridine disulfide (PDS) group and around 10 azide groups per polymer chain which is enough for post-conjugation. Keep in mind that the azide group is not stable when exposed to high temperatures (70 °C) for long times due to side reactions such as cycloaddition between azido and methacryl groups during the polymerization process [12].

2. If different kinds of cationic polymers containing azide groups or functional peptides will be applied to this protocol, make sure there are no free thiol groups in the polymer chain and peptide sequences, as the free thiols could compete with azide groups to react with the BCN groups due to thiol-yne addition [13].
3. Always sterilize the buffers and solutions by filtration through 0.2-μm sterile filters after preparation.
4. Because the carbonate ester in the polymer side chain of HPMA-DMAE is prone to hydrolysis and lose the DMAE cationic charge groups in neutral ($t_{1/2}$ ~ 10 h, 37 °C) and alkaline pH and is rather stable at pH 5 [9], the polymer stock solution was prepared in acetate buffer pH 5.0.
5. BCN-PEGylation of protected GALA peptide fragments can help preventing side reactions of amine-NHS reaction and achieve a relatively good BCN-PEG-GALA yield (~40%). For example, with side chain protected GALA, we can prevent BCN-PEG-NHS from reacting with the histidine's imidazole side chains to form carbonyl imidazolide. Besides, our attempts of solid-phase PEGylation have resulted in very low coupling efficiency (<1%). We speculate that the molecular size of BCN-PEG-NHS (~5000 Da) is too big to sufficiently penetrate into the solid-phase polymer mesh, thereby significantly lowering the chance of NHS-amine reaction.
6. To investigate the purity of conjugated GALA peptide, we recommend cleaving a portion of on-resin peptide and determining its purity using high-performance liquid chromatography (HPLC) characterization (UV detection 280/220 nm).
7. Ethanedithiol (EDT)-containing cleavage cocktails (e.g., reagent B) should be avoided as to prevent BCN group reduction.
8. The success of conjugation of GALA to the BCN-PEG is confirmed using ^{1}H nuclear magnetic resonance (NMR) and HPLC analysis.

9. During mRNA polyplex preparation, use pipetting instead of vortexing to avoid potential shearing of the mRNA.
10. For each new batch of polymer, performing an agarose gel electrophoresis and measuring mRNA-containing polyplex size at varying N/P ratios are necessary and important. Based on these results, an optimal N/P ratio can be determined at which no free mRNA can be discerned in the electrophoresis gel and polyplexes with an average size <300 nm are obtained. At this optimal N/P ratio, excess of free polymer inside the polyplex solution is avoided as free polymer will compete with BCN-PEG-GALA conjugation to the polyplexes, thereby lowering peptide post-conjugation efficiency.
11. To speed up the setting of the gel, this step can also be performed in a cold room.
12. To efficiently conjugate pre-formed mRNA polyplexes with BCN-PEG-GALA in the presence of free polymer chains dissolved in the polyplex dispersion, it is very important to consider the ratio of cationic polymer, mRNA and BCN-PEG-GALA peptides. Since GALA has seven glutamic acid residues in its sequence and is negatively charged at neutral pH (*see* Fig. 1), a change in ratio between polyplexes and BCN-PEG-GALA may change the total surface charge of the formed mRNA polyplexes, leading to changes in cellular uptake and subsequent endosomal escape efficiency. This method describes GALA post-modified mRNA polyplex formation at a ratio that gives highest mRNA transfection on D1 cells in culture.
13. Unlike EGFP-mRNA, for fluorophore labeled Cy5-EGFP-mRNA, significant polyplex aggregation was observed at a final RNA concentration higher than 150 μg/mL, possibly due to the hydrophobic nature of the Cy5 dye.
14. The average size of the polyplex population can be measured with dynamic light scattering (DLS) with a diluted mRNA concentration 10 μg/mL in HEPES buffer as a negative control. The average size should be around 120 nm.
15. The BCN-PEG-GALA is added to pre-formed mRNA polyplexes at a (BCN in BCN-PEG-GALA)/(N_3 in pHDPA) molar ratio of 0.6. This ratio gives the highest transfection in dendritic cells and macrophages in an EGFP_mRNA transfection experiment [5].
16. For monitoring the conjugation process of BCN-PEG-GALA to polyplexes, the zeta potential of the polyplexes can be continuously measured using a Malvern Zetasizer Nano-Z (Malvern, UK) with universal ZEN 1002 "dip" cells and at 25 °C. The zeta potential of the polyplexes will decrease as a function of time due to shielding of the surface charge of the polyplexes

by the coupling of BCN-PEG-GALA, and the zeta potential will reach a plateau after 2 h incubation [8].

17. The amount of DTT added corresponds with a half molar equivalent to PDS groups of the polymer used in particle formation to induce self-crosslinking of the polyplexes via thiol-disulfide exchange reaction [14]. The sequence of adding of DTT after the BCN-PEG-GALA step is very important, as free thiol groups could react with BCN groups resulting in a lower GALA post-conjugation and loosely crosslinked, unstable polyplexes.
18. Freeze-drying will also increase the azide-BCN conjugation efficiency [8]. Polyplexes can be stored at 4 °C for 1 week and at −20 °C for a month with no functional loss as was verified by us by determining the cell transfection efficiencies over storage time. The resulted GALA modified mRNA polyplexes with a size around 150 nm and zeta potential of −8 mV. The particles size increased from 120 nm to 150 nm which was due to the successful conjugation of BCN-PEG-GALA and freeze-drying.

Acknowledgments

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