

Transferrin ornamented thymoquinone loaded PEG-PLGA nanoparticle furnishes anticarcinogenic effect in non-small cell lung carcinoma through modulation of miR-34a and miR-16

Priyanka Upadhyay¹, Sushmita Sarker¹, Avijit Ghosh¹, Payel Gupta², Shaswati Das¹, Manisha Ahir¹, Saurav Bhattacharya¹, Sreya Chattopadhyay², Swatilekha Ghosh³, Arghya Adhikary^{1}*

¹Center for Research in Nanoscience and Nanotechnology, Technology Campus, University of Calcutta, JD-2, Sector III, Salt Lake City, Kolkata-700106, India.

²Department of Physiology, University of Calcutta, 92 Acharya Prafulla Chandra Road, Kolkata, 700009, WB, India

³Amity School of Biotechnology, Amity University, Kolkata. Major Arterial Road (South-East), Action Area II, Newtown, Kolkata, West Bengal 700135, India.

Table 1: mi-RNA Primer sequences

Primer Name	Primer Sequence
Has-miR -U6 RT	5' - AAA ATA TGG AAC GCT TCA CGA ATT TG - 3'
Has-miR -U6 Forward	5' - CTG GCT TCG GCA GCA CAT A - 3'
Has-miR- U6 Reverse	5' - CAC GAA TT GCG TGT CAT CC -3'
Has -miR-34a-5p Stem	5' - GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG
Has-miR-34a Forward	CAA CC- 3'
Has-miR-16 Stem	5' - CAG GCA TGG CAG TGT CTT AGC T -3'
Has-miR-16 Forward	5' - GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG
Universal Reverse	ACC GCC AA - 3'
pre-miR-16-1 Forward	5' - CCC CCC TAG CAG CAC GTA - 3'
pre-miR-16-1 Reverse	5' - CCA GTG CAG GGT CCG AGG TA - 3'
	5' - CCT TGG AGT AAA GTA GCA GCA CAT AAT G - 3'
	5' - ATA TAC ATT AAA ACA CAA CTG TAG AGT ATG - 3'

Table 2: m-RNA Primer sequences

Primer Name	Primer Sequence
Human 18S Forward	5'- GTA ACC CGT TGA ACC CCA TTC GT - 3'
Human 18S Reverse	5'- CCA TCC AAT CGG TAG TAG CGA CGG - 3'
Human P53 Forward	5'- CTT TGA GGT GCG TGT TTG TG - 3'
Human P53 Reverse	5'- AGA GGA GCT GGT GTT GTT G - 3'
Human Bax Forward	5'- GGG TGG TTG GGT GAG ACT C - 3'
Human Bax Reverse	5'- AGA CAC GTA AGG AAA ACG CAT TA - 3'
Human Bcl2 Forward	5'- GTG GAT GAC TGA GTA CCT GAA C - 3'
Human Bcl2 Reverse	5'- GCC AGG AGA AAT CAA ACA GAG G - 3'
Human GAPDH Forward	5'- CTT TGG TAT CGT GGA AGG ACT C - 3'
Human GAPDH Reverse	5'- GTA GAG GCA GGG ATG ATG TTC - 3'
Chick GAPDH Forward	5' - GAG GAA AGG TCG CCT GGT GGA TCG - 3'
Chick GAPDH Reverse	5' - GGT GAG GAC AAG CAG TGA GGA ACG - 3'
Human Alu Forward	5' - ACG CCT GTA ATC CCA GCA CTT - 3'
Human Alu Reverse	5' - TCG CCC AGG CTG GAG TGC A - 3'
Chick GAPDH Forward	5' - GAG GAA AGG TCG CCT GGT GGA TCG - 3'
Chick GAPDH Reverse	5' - GGT GAG GAC AAG CAG TGA GGA ACG - 3'

Synthesis:**Synthesis of n-hydroxysuccinimide activated PLGA copolymer:**

10 g of PLGA polymer (PLGA 50:50, RG 503H, molecular weight 28 kDa) was taken in 10 mL of dry dichloromethane. Subsequently, 270 mg of n-hydroxysuccinimide (NHS) (2.34 mmol) and EDC 230 mg (2.39 mmol) were added to the PLGA solution and the mixture was stirred at room

temperature for 16 hours under a argon atmosphere. After completion of the reaction, the PLGA-NHS was precipitated with cold di-ethyl ether and centrifuged at 8000 rpm for 20 minutes at 4°C. The procedure was repeated 3 times to complete removal of any unreacted starting materials. The white powder obtained was then dried over vacuum and stored at -20°C.

Synthesis of amine functionalized PLGA copolymer:

For synthesis of PLGA-PEG amine, pre activated PLGA-NHS 1 g was dissolved in 10 mL of dichloro-ethane (DCE), and 2,2'-(Ethylenedioxy)bis(ethylamine) 148 mg (1 mmol) was added to the solution. The mixture was stirred at room temperature for 24 hours. After completion of the reaction, the product was precipitated with ice-cold ether and centrifuged at 8000 rpm for 20 minutes at room temperature. The precipitate was washed three times with ice-cold methanol to remove any unreacted PEG diamine. It was then re-dissolved in acetone-dichloromethane mixture (1:1) and further precipitated in cold diethyl ether. Finally, the product was dried in vacuum and storage at -20°C.

Synthesis of thymoquinone loaded amine functionalized PLGA copolymer Nanoparticle: (PLGA-PEGNH₂@TQ NPs)

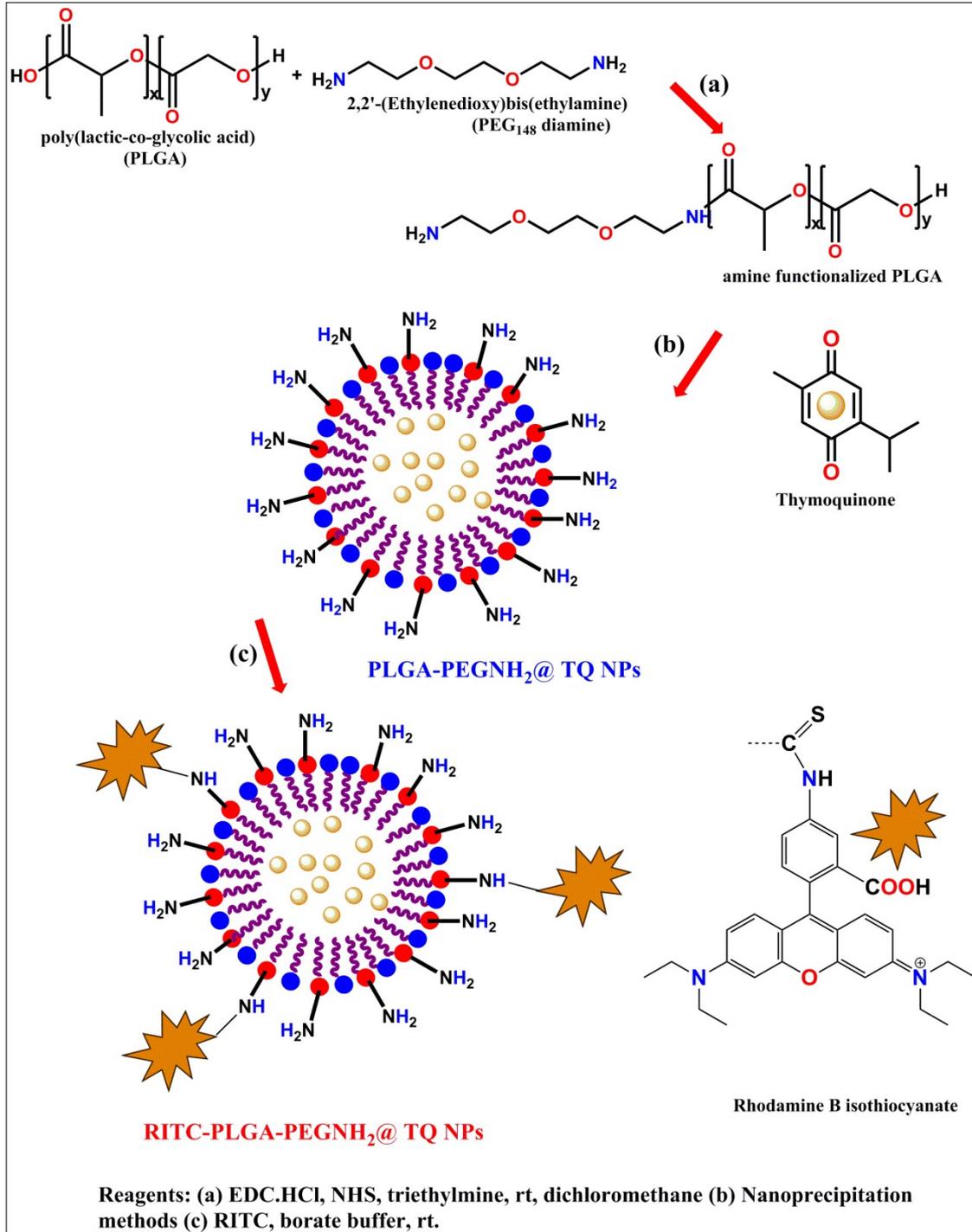
Nanoparticles were prepared using a same method used for PLGA-PEG NPs.

100mg of Polyethylene glycol (m.w: 4000) was dissolved in 10 ml of Milli-Q water at 37°C in an Ultrasonic Bath Sonicator. Then 10ml of 4% polyvinyl alcohol (PVA) solution was added and the mixture was homogenized in a Probe Sonicator /Ultrasonic Homogenizer for 2min to obtain a clear solution. Next, 100 mg of amine functionalized PLGA copolymer was dissolved in 2 ml of dichloromethane as an oil phase at room temperature to obtain uniform solution. The amine factionalized PLGA in dichloromethane was added drop wise by a syringe to the above PEG4000 solution (containing PVA) at 40°C with continuous stirring at 5000 rpm. Then it was further emulsified in same probe sonicator for additional 4 min. After then, the mixture was stirred at 2000 rpm for 4h to remove organic solvent. In the next step, 60 mg thymoquinone

dissolved in 5ml of dichloromethane was added by a 1ml syringe in spraying methods into the previous emulsion at 40°C with a flow rate of 0.2 ml/min for 30 min at continuous mode in Probe Sonicator. After complete injection of thymoquinone by spraying, the organic solvent present in suspension was evaporated by room temperature stirring the whole solution at 1000 rpm for overnight at dark. The nanoparticles were then collected by centrifugation at 8000g G for 30 min at 4°C. Then the precipitate was washed with cold ethanol and Milli-Q water.

Conjugation of Rhodamine B isothiocyanate (RITC) with PLGA amine NPs: (RITC-PLGA-PEGNH₂@TQ NPs)

In the final step conjugation of the resulting NPs with Rhodamine B isothiocyanate (RITC) was carried out. 100 mg of NPs was suspended in 3 mL of borated buffer (50 mM, pH 8.5) or (0.15 M, NaHCO₃/ Na₂CO₃, pH 9.0). Then, 5.36 mg of RITC (0.01 mmol) was added into the suspension and stirred at room temperature in the dark for 24 hours. After completion of the reaction, the reaction mixture was quenched with water to obtain a dark pink solid precipitate. The solids ppt was collected by centrifugation. Then it was washed with water and dried under high vacuum.



Scheme S-1: Synthetic route to RITC labeled TQ nanoparticles.