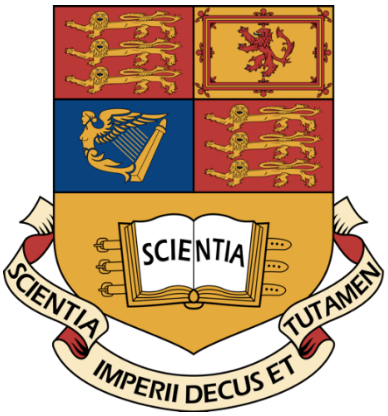
Development of Serum-stable Ternary Complexes for Nucleic Acid Delivery



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Abstract

Polyplex nanoparticles of cationic polymers (e.g. pABOL) and plasmid DNA (pDNA) are commonly used for nucleic acid delivery in preclinical research. However, positively charged polyplexes aggregate with negatively charged serum proteins and extracellular matrix components when injected *in vivo*, leading to local toxicity and restricted biodistribution. In order to improve polyplex transport and enable wider applications of polymers in nucleic acid delivery, we coated polyplexes of poly(CBA-co-4-amino-1-butanol (ABOL)) (pABOL) and pDNA with negatively charged poly(α-L-glutamic acid) (α-PGA) or polyethylene glycol grafted PGA (PEG-g-PGA) to form ternary complex nanoparticles (TCNs). Coating with PGA or PEG-g-PGA was able to decrease the TCN's zeta potential to neutral or negative surface charge without interfering with polyplex structure at various coating polymer: polyplex formulation ratios. Competitive heparin binding studies and DNase I protection assays were used to evaluate the nucleic acid packaging stability of polyplex and TCN formulations via gel electrophoresis and fluorescence quantification assay. High throughput dynamic light scattering (DLS) measurements were further used to longitudinally evaluate the size stability of various TCN formulations in several aggregation-prone environments during incubation at 37 °C. Finally, delivery of pDNA encoding GFP to HEK293T cells was used to compare the *in vitro* transfection potential of polyplexes and TCNs (PGA) formulated with and without PEG in both serum-free and serum-containing medium. We show that PGA only can coat pABOL polyplexes without unpackaging the encapsulated pDNA, but PEG-g-PGA may lead to disassociation of polyplexes at low coating polymer: polyplex formulation ratios. TCNs coated with PEG-g-PGA are less prone to aggregation in media with physiological saline and serum concentrations. Intriguingly, the *in vitro* transfection efficiency of TCNs (PGA) was greater than that of polyplexes, while TCNs (PEG-g-PGA) demonstrated much lower efficiency in serum free medium, likely due to lower cellular uptake because of steric shielding. These results collectively demonstrate that TCN formulations with PGA or PEG-g-PGA can enhance polyplex stability and differentially alter transfection efficiency without compromising nucleic acid packaging. Future *in vivo* transfection studies are needed to compare TCN (PGA) and TCN (PEG-g-PGA) formulations, as it is well known that *in vitro* and *in vivo* uptake of PEGylated nanomedicines differs greatly.

Key words: poly(α-L-glutamic acid); PEGylation; Ternary complexes ; pABOL; nucleic acid vaccines

Acknowledgement

I would like to acknowledge Dr. Fabio Fisher for the gift of mRNA, Prof. Robin Shattock for the gift of saRNA, and Lijun Hu for assistance with experiments. I am very grateful to David for his support and guidance in completing this project and very thankful to Stevens team. Finally, I would like to express my special thanks to Molly who gave me the opportunity to work in her lab.

List of abbreviations

pDNA	Plasmid DNA
mRNA	Messenger RNA
siRNA	Small interfering RNA
saRNA	Self-amplifying RNA
APCs	Antigen-presenting cells
MPS	Mononuclear phagocyte system
RES	Reticular endothelial system
PEI	Polyethylenimine
PAMAM	Polyamidoamine
pABOL	poly(CBA-co-4-amino-1-butanol (ABOL))
GSH	Glutathione
LNs	Lymph nodes
sgRNA	single guide RNA
α-PGA	poly(α-L-glutamic acid)
PEG	Polyethylene glycol
PEG-g-PGA	polyethylene glycol grafted PGA
TCNs	Ternary complex nanoparticles
MW	Molecular weight
GPC	Gel permeation chromatography
NMR	Nuclear Magnetic Resonance
DMSO	Dimethyl sulfoxide
GFP	Green florescent protein
LB	Luria-Bertani
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
PDI	Polydispersity index
TAE	Tris-acetate EDTA
TE	Tris-EDTA
EDTA	Ethylenediaminetetraacetic acid
DMEM	Dulbecco's Modified Eagle Medium
HEK	Human embryonic kidney

Contents

1. Introduction

1.1 Nucleic acids as vaccines

Since 2020, two messenger RNA (mRNA) vaccines have been clinically approved, which is a major milestone in non-viral and viral nucleic acid delivery in humans. (Jafari et al., 2022) Nucleic acids which encode antigens in pDNA, and mRNA have shown promise in both infectious disease prevention and cancer therapy. (Guan & Rosenecker, 2017) In order to prevent infectious diseases, nucleic acid vaccines mimic viral use of host cell transcription and translation mechanisms to produce encoded antigen polypeptide, which is then processed by antigen-presenting cells (APCs) of the innate immune system. Nucleic acids also amplify the innate immune response to vaccination by direct binding to danger signal receptors in APCs, thus serving as “self-adjuvants” and promoting potent antigen-specific antibody and cytotoxic T-lymphocyte adaptive immune responses. (Vogel & Sarver, 1995) In other applications, nucleic acids can correct a disease-related inherited gene by adding exogenous pDNA or mRNA, knocking down gene expression (siRNA), or altering genomic DNA (e.g. Cas9/sgRNA). (E et al., 2018; H & Philip, 2016) However, to achieve such functions, nucleic acids need to be delivered to specific subcellular compartments within cells that reside in specific tissues within body. This is a challenging task because nucleic acids face many obstacles during delivery, namely (1) extracellular instability: naked nucleic acids are degraded by nucleases within minutes, phagocytosed by mononuclear phagocyte system (MPS), and cleared rapidly by reticular endothelial system (RES) and (2) intracellular barriers: poor translation efficiencies due to poor uptake, trafficking into lysosomes with degradative enzymes, and transport back out of cells by exocytosis. (Kutzler & Weiner, 2008; Pack et al., 2005) Any failure during delivery eliminates the ability to induce immune responses. Therefore, there is a need for a drug delivery technology which can protect and transport nucleic acids away from the immediate vicinity of injection while still achieving potent intracellular translation.

1.2 Nanoparticles for lymphatic targeting

Both traditional and nanoparticulate vaccines (“nanovaccines”) achieve enhanced efficacy when administered intradermally or subcutaneously by targeting APCs residing in the skin or lymph nodes. (Ma et al., 2011; Mohanan et al., 2010). APCs can capture vaccine antigens from skin tissues, and then migrate into lymph nodes (LNs), where APCs present the processed antigen peptides to T cells and B cells, triggering adaptive immune responses.(Swartz, Hubbell & Reddy, 2008; Ma et al., 2011, 2010) Moreover, APCs can regulate both innate and adaptive immunity by releasing cytokines and chemokines upon activation. (Reis e Sousa, 2004; Ma et al., 2010) LNs contain many

resident DCs which specialize in the capture and presentation of antigens that freely drain into LNs, indicating that vaccine drainage to DCs in LNs can enhance protective immune responses. Analogous to past work on protein-based vaccines, we therefore seek to enhance the LN accumulation of polymeric nucleic acid vaccine nanoparticles.

There are some important criteria that nanoparticles must meet in order to drain to LNs. For example, (1) Small size, as previous studies show that the suitable size of nanoparticles for lymphatic uptake is between 10 and 100 nm. Small particles rapidly drain to the LNs in which they can be captured by resident DCs, however, particles larger than 100 nm rely on the cellular transport of DC migrating from skin. Moreover, endothelial cell junctions (initial lymphatic vessels) work as molecular sieves and stop large nanoparticles from freely entering the afferent lymphatics. (Manolova et al., 2008) Reddy et al found that 20 and 45 nm poly(ethylene glycol)-stabilized poly(propylene sulfide) (PEG-PPS) nanoparticles have good retention in LNs. (Reddy et al., 2006). (2) Balance between hydrophobicity and hydrophilicity, as highly hydrophobic nanoparticles may aggregate at the injection site and thus decrease LN drainage, while hydrophilicity nanoparticles have poor cellular uptake. In this case, to balance the hydrophobicity and hydrophilicity of nanoparticles, the PEG density is an important parameter. PEGylation is required for lipid nanoparticle (LNP) function, both by enabling stable self-assembly and by promoting LN drainage. For example, PEGylating cationic liposomes not only accelerated their drainage into LNs, but also enhanced their retention time in LN and liposome uptake.(Zhuang et al., 2012) Previous studies showed that increasing the length of PEG chains significantly reduced cellular uptake and activation of DC while shortening the PEG chains enhanced DC activation but meanwhile increased cytotoxicity.(Waku et al., 2019) (3) Neutral or negative surface charge, as nanoparticles with neutral or negative surface charge can promote lymphatic drainage after subcutaneous injection. (Zeng et al., 2017)

1.3 Lipid nanoparticles for nucleic acid vaccine delivery

Many different of platforms have been developed for nucleic acid vaccine delivery, such as lipids, lipid-like materials, and different kinds of polymers such as polylysine, polyethylenimine (PEI) and polyamidoamine (PAMAM).(Hajj & Whitehead, 2017; Meng et al., 2021; Kowalski et al., 2019) So far, only LNPs have been clinically approved as mRNA COVID-19 vaccines. (Baden et al., 2020) As a nucleic acid vaccine delivery platform, LNPs have lots of benefits, for example, (1) LNPs can form stable nanostructures and protect nucleic acids from degradation. (Kim et al., 2021) (2) LNPs can be internalized by endocytosis upon reaching target cells. (Li, Zhang & Dong, 2019;

Kim et al., 2021) (3) LNPs are relatively easy to synthesize by rapid mixing and have reproducible and scalable production with high encapsulation efficiency and homogeneous size distribution. (Ilya et al., 1998) Most importantly, LNPs can target APCs at both the site of administration and draining LNs and achieve rapid translation of vaccine antigen no matter which administration route (intradermal or intramuscular). (Liang et al., 2017) However, LNPs have many limitations: (1) LNPs cannot be stored for a long time and should be stored in very cold frozen conditions (-80 °C) and injected directly after thawing.(Kim et al., 2021) (2) LNPs' lipid components can directly activate the immune system. For examples, PEG-lipids could stimulate the complement system and then induce hypersensitivity reactions.(Knop et al., 2010; Abu Lila, Kiwada & Ishida, 2013) (3) LNPs may cause cytotoxicity and the induction of pro-inflammatory factors depending on the dose, lipid properties and cell types. (Lv et al., 2006; Filion & Phillips, 1997; Dokka et al., 2000; Sedic et al., 2018) (4) Ionizable LNPs can only trigger specific type of immune response which restricts their application to IL-6 dependent processes(Li et al., 2022; Tahtinen et al., 2022) and (5) Transfection efficiency is still quite low (expression for days in a small percentage of LN-resident cells).(Kim et al., 2021)

1.4 Polymeric delivery of nucleic acid vaccines

Polymeric material design enables the application of diverse chemistries and macromolecular structures to generate more sophisticated non-viral nucleic acid delivery vectors. (Tang & Szoka, 1997; Filion & Phillips, 1998) Polymeric vectors have intrinsically greater stability than monomeric LNPs as a result of their multivalent charge-charge interactions with nucleic acids, enabling robust adaptability to various storage conditions and greater *in vivo* longevity. (Pack et al., 2005) Polymers such as PEI, PAMAM and poly(CBA-co-4-amino-1-butanol (ABOL)) (pABOL) are already widely used for nucleic acid delivery. (Taranejoo et al., 2015; Neu et al., 2007; Xia et al., 2016; Blakney et al., 2020) PEI has a high density of secondary amines which mediate efficient endosomal escape through the 'proton-sponge' effect and subsequently high transfection efficiency.(Mini & M, 2002) However, PEI is extremely cytotoxic.(Taranejoo et al., 2015) pABOL is a linear, bioreducible polycation which is less toxic than PEI because its internal disulfide bonds are degraded in the cytosol by intracellular glutathione (GSH), rapidly lowering its molecular weight (MW) and cytotoxic non-specific crosslinks. Compared to other biodegradable polycations, pABOL has good water solubility and stability against hydrolysis.(Lin et al., 2007) The Stevens and Shattock groups have shown that pABOL achieves significant protein expression from self-amplifying RNA (saRNA) when injected intramuscularly or intradermally in mice. (Blakney et al., 2020). Furthermore, a comparison of pABOL/saRNA polyplexes and LNP formulations has

been done to characterize the protein expression and immunogenicity. (Blakney et al., 2021) This study showed that although pABOL and LNP formulations exhibit similar size and encapsulation efficiency, pABOL formulations have positive zeta potential while LNP formulations have neutral zeta potential. They also found that pABOL formulations can increase protein expression in vivo relative to LNP. The differences in surface charge could have an impact on the immunogenicity, as previous studies showed that surface charge can determine the biodistribution of nanoparticles and nearly neutral or slightly negative charged particles could express exclusively splenic signal and have potential to deliver antigens to lymphoid-resident DCs. (Kranz et al., 2016) This indicates that changing polyplex's surface charge to neutral or negative could shift polyplex's biodistribution to the LNs and thus enhance vaccine immunogenicity.

Conjugating polycations with polyethylene glycol (PEG), also known as PEGylation, is commonly used to improve the stability and transport of polyplexes.(Suk et al., 2016) However, PEGylation cannot fully neutralize surface charge, and the ultimate gains in transport efficiency achieved by these approaches are limited. Another method for modifying the surface charge of polyplexes is the addition of a third, negatively charged polymer to form ternary complex nanoparticles (TCNs). Thus, we hypothesize that adding a coating polymer to pABOL polyplexes will preserve the nucleic acid packaging and endosomal escape attributes of the underlying polyplex while fully reprogramming its surface charge and chemistry to enable greater LN drainage and improved immunogenicity**.**

1.5 Polyanions for nucleic acid vaccines delivery

Polyglutamic acid (PGA) is a hydrophilic, biodegradable polypeptide commonly used in drug delivery because of its negative charge and biocompatibility. (Ikeda et al., 2018; Sahdev, Ochyl & Moon, 2014) The highly anionic nature of PGA can be used to reduce the surface charge of polyplexes, thus reducing cytotoxicity, enhancing shelf stability, and preventing aggregation mediated by serum proteins. (Trubetskoy et al., 2003; Ito, Iida-Tanaka & Koyama, 2008; Mokhtarzadeh et al., 2016; Hu et al., 2017). In addition, the carboxylic acid side chains of PGA can be easily conjugated to other molecules to covalently modify particle surface chemistries.(Ikeda et al., 2018) Previous studies have shown that TCNs formed with γ-PGA have increased cellular uptake by γ-PGA receptor-mediated processes and increased the transfection efficiency of pDNA polyplexes even in the serum-containing medium.(Ito et al., 2010; Kurosaki et al., 2009; Wang et al., 2010) For comparable TCNs coated instead with poly(α-L-glutamic acid) (α-PGA), the reason for observed enhanced transfection efficiency and cellular uptake in serum is still

unknown. Dekie and his co-workers found that α-PGA with pDNA and some cationic polymers can self-assemble into TCNs, be stable in the presence of serum, protect pDNA from DNase degradation, and enhance uptake *in vitro*. (Dekie et al., 2000) Salmasi et al used PEI and α-PGA to synthesize DNA/PEI/α-PGA triplexes and characterized size, zeta potential, cytotoxicity, in vitro and in vivo transfection efficiency. As a result of coating with α-PGA, the cytotoxicity of PEI/DNA polyplexes decreased and the transfection efficiency in the presence of serum increased without change of size and structure. (Salmasi et al., 2018) Overall, the benefits of using α-PGA as a polyplex coating polymer include: (1) altered polyplex biodistribution without unpackaging; (2) enhanced transfection efficiency in serum-containing environments; (3) decreased cytotoxicity of polyplexes. These benefits have motivated us to use α-PGA to modify the surface charge of the Stevens group's pABOL polyplexes, which may help to enhance the LN drainage and improve immunogenicity of this promising saRNA vaccine delivery vehicle.

1.6 Effects of PEGylation and PEG density on TCN delivery

PEGylated polyanions create a hydrophilic protective layer for TCNs, reducing non-specific interactions with serum proteins and increasing the colloidal stability of TCNs under physiological conditions. Previous studies have shown that surface PEGylation has been widely used for the neutralization of siRNA nanocarriers to reduce phagocytosis and increase stealth from MPS following systemic administration. (Mishra, Webster & Davis, 2004a; Venkataraman et al., 2011; Sato et al., 2007) Benns et al found that PEGylation significantly reduces the cytotoxicity and thus improves the biocompatibility of complexes. (Benns, Mahato & Kim, 2002) However, adding PEGylated polyanions to polyplexes may loosen the compact pDNA complexes, which has been hypothesized to enhance transcriptional efficiency but may also increase susceptibility to nuclease-mediated degradation. (Koyama et al., 2006; Ito et al., 2010; Yue et al., 2012) Polyanion PEGylation density also impacts TCN structure. Werfel et al found that optimally PEGylated TCNs can achieve colloidal stability and neutral surface charge but higher cellular uptake than more densely PEGylated particles.(Werfel et al., 2017) Mishra et al also noted that a high degree of TCN PEGylation may reduce cellular uptake. (Mishra, Webster & Davis, 2004) In this case, the grafting density of PEG must be tuned to balance colloidal stability and cellular uptake. Thus, we leveraged differentially grafted PEG-PGAs as coating polymers to balance extracellular stability and LN transport of pABOL TCNs. After demonstrating TCN stabilization, we will conjugate targeting ligands to PEG before PGA conjugation to increase uptake in target APC populations (e.g. LN resident DCs).

1.7 Hypothesis and aims

Cationic polymers can be used for nucleic acid delivery but with cytotoxicity and undesirable interactions with serum proteins, which may have negative effects on formulation stability, cellular uptake, and transfection efficiency. (Modra et al., 2015) To solve this problem, a third negatively charged polymer is introduced to form TCNs with neutral or negative surface charge. (Kurosaki et al., 2009) TCNs may enable less stringent storage conditions and lower dosing to mitigate the drawbacks of current LNP delivery systems and positively charged polyplexes. Although many studies have shown that adding a third negatively charged polymer to form TCNs can enhance stability, α-PGA has never been characterized as a coating polymer for pABOL polyplexes. Overall, the novelty of this study lies in the development of a nucleic acid vaccine delivery platform using medium MW α-PGA with different percentages of PEG grafted α-PGA (PEG-g-PGA) as coating polymers to form TCNs with small size, neutral or negative zeta potential, good colloidal stability, high cellular uptake, and high transfection efficiency. **We hypothesize that optimizing the surface chemistry of pABOL TCNs will enable nucleic acid vaccine delivery to APCs with high efficiency by improving on polyplex stability and transport properties.**

The specific aims of this study are as follows:

(1) Synthesize differentially grafted PEG-g-PGA.

To investigate the impact of PEG density on TCN properties, PGA carboxylic acids were grafted with different amounts of PEG-NH_2 (2.5, 5, 10 mol%) via EDC/NHS amidation.(Boulmedais et al., 2004) Synthesized PEG-g-PGA coating polymers were analyzed by aqueous GPC (gel permeation chromatography) and NMR (Nuclear Magnetic Resonance) to assess polydispersity and purity.

(2) Optimize formulation parameters of polyplexes and TCNs to enable LN drainage.

To achieve LN drainage, the size of TCNs and polyplexes should be within 50-100 nm with nearly neutral or slightly negative charge. TCN should have good biocompatibility, low cytotoxicity and protect nucleic acids from degradation.(Zeng et al., 2017) Formulation parameters such as the mixing method, mixing volume ratios, material charge ratios, and different buffer systems with various pH were investigated to optimize TCNs with these properties.

(3) Characterize polyplex and TCN formulations in terms of size, zeta potential, nucleic acid packaging efficiency, stability, and transfection efficiency in vitro.

The impact of PGA or PEG-g-PGA on TCN characteristics including size, zeta potential, condensation properties, stability and transfection efficiency was investigated. The hydrodynamic diameter and zeta potential of TCNs and polyplexes were analyzed by dynamic light scattering (DLS) and electrophoretic mobility (Zeta), respectively. Nucleic acid condensation properties were studied through DNase I protection assay, gel electrophoresis, and fluorescence quantification assays. Finally, the transfection efficiency of polyplexes and TCNs was assessed in media both with and without serum in the HEK293T cell lines by epifluorescence microscopy.

2. Materials and Methods

2.1 Materials

Low (3-5 kDa) and medium (15 kDa) MW α-PGA were purchased from Alamanda. mPEG5k-NH2 was purchased from Biochempeg. Sulfo-NHS (N-hydroxysuccinimide), EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and 10000X Gel red were purchased from Sigma-Aldrich. SnakeSkin™ Dialysis Tubing (10K MWCO, 22 mm), Quant-iT™ PicoGreen™ dsDNA Assay Kits and DNase I solution (1Unit/μL), phosphate buffer, agarose, and TAE (Tris-acetate-EDTA) buffer were purchased from ThermoFisher. mRNA was a gift from Dr. Fabio Fisher and saRNA was a gift from Prof. Robin Shattock. pCAG-GFP pDNA was purified by PureLink™ HiPure Plasmid Filter Maxiprep Kit from ThermoFisher. 8kDa pABOL was previously synthesized by Dr. Jonathan Yeow in the Stevens Group.

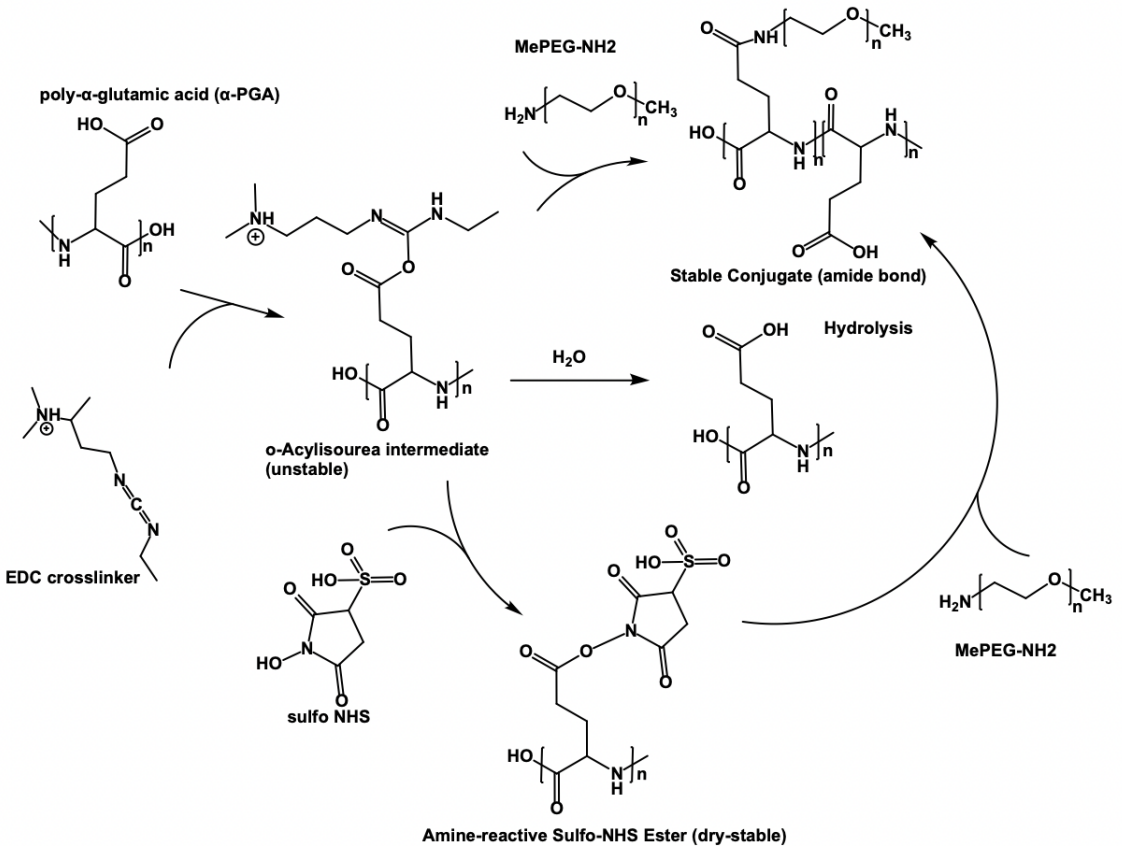
2.2 Grafting of mPEG-NH2 to PGA

A series of experiments was performed to optimize amidation reaction conditions for mPEG-NH_2 grafting to PGA. Initial experiments were conducted in alkaline aqueous buffer (50mM Borate buffered saline (BBS) buffer pH 8.0). Different grafting percentages of PEG-g-PGA were achieved by adding different amounts of PEG to the reaction system. 20 mg (0.13 mM) MMW PGA (15 kDa), various of mass of mPEG5k-NH_2 depending on the weight ratio of PGA and PEG (1, 2.5, 10 mol% relative to PGA COOH), and 1.08mg (0.5 mmol) of NHS were dissolved in 1ml of BBS. Then, 5.9mg EDC (3.75 mmol) was dissolved in the mixture with stirring.

Follow-up experiments were conducted in a mixed aqueous-organic solvent solution to reduce premature hydrolysis of the unstable intermediate reaction products, illustrated in **Scheme 1**, which decreases the conjugation reaction efficiency. Anhydrous dimethyl sulfoxide (DMSO) was used to reduce premature hydrolysis of EDC before the coupling reaction could proceed. 41.67 mg (8.33 mM; 2.5%), 83.33 mg (16.67 mM; 5%) and

166.67 mg (33.33 mM; 10%) of mPEG5k-NH_2 were mixed separately with 50mg (0.02mM) MMW PGA in 2ml, 2.8ml and 4.33ml BBS buffer (to maintain the same concentration). EDC and sulfo-NHS were dissolved in DMSO at 50mg/mL and 65 mg/mL as stock solution. Then, 113.8 uL (5.69 mg, 0.74 mM) EDC and 556.7 uL (36.19 mg, 166.67 mM) sulfo-NHS stock solutions were added to the reaction.

All reactions proceeded for 6 hours at room temperature. Then, reaction mixtures were dialyzed (MWCO 10 kDa) for 24 hours against phosphate buffer (4L, 0.1M, pH=7.4, Na_2HPO_4/NaH_2PO_4) and against 4L of deionized water for 24 hours more before lyophilization. Purified PGA-g-PEG was analyzed by ^{1}H NMR (JEOL) and aqueous GPC (Malvern). Percent grafting and theoretical polymer MW were calculated from ^{1}H NMR (D_2O) spectra recorded by a 400 MHz spectrometer at room temperature. The monodispersity of conjugated polymer was determined by aqueous GPC using 200 mM $NaNO_3$, 3 mM NaH_2PO_4 and 7 mM Na_2HPO_4 at pH 7.4 as the mobile phase. All samples were filtered through a 0.22 μm syringe before injection.



Scheme 1. mPEG2k-NH2 and MMW PGA conjugation via EDC and NHS amidation.

2.3 Preparation of plasmid DNA (pDNA)

Plasmid DNA encoding green fluorescent protein (GFP) gene was obtained from Escherichia coli strain producing pCAG-GFP grown in ampicillin Luria-Bertani (LB) agar. A single colony was picked from the transformation plate with a pipette tip and put in a round-bottom tube with 1 mL LB media with ampicillin for 6 hours. After the day culture reached an optimal density, it was then diluted 1000X into 500 mL Amp-LB in a 2 l Ehrlenmyer flask and shaken at 250 rpm at 37 °C. After 16 hours, the overnight culture was pelleted by spinning at 5000 rcf for 5 minutes at 4 °C and pDNA purified by maxi-prep kit according to the manufacturer's instructions. The concentration and purity of purified pDNA was determined by UV absorbance at 260nm, and by 1.0% agarose gel electrophoresis at 110 V for 30 minutes following linearization by EcoRI restriction digest.

2.4 Preparation of polyplexes (PP) and ternary complex nanoparticles (TCN)

Methods for polyplex and TCN formulation are illustrated in **Scheme 3**. Firstly, pDNA and pABOL (8kDa) stock solutions were made at 1 mg/mL and 50 mg/mL separately in 20 mM Bis Tris (pH 6.5) and 20 mM HEPES (pH 7.0 and 7.4) buffers. The concentration of pDNA was adjusted via Nanodrop quantification. The charge ratio indicates the molar ratio of number of ionizable groups in polymers and nucleic acids. The amount of pABOL and pDNA was calculated according to Eq.1 based on the N (molarity of positively charged pABOL amines) to P (molarity of negatively charged DNA phosphates) ratios.

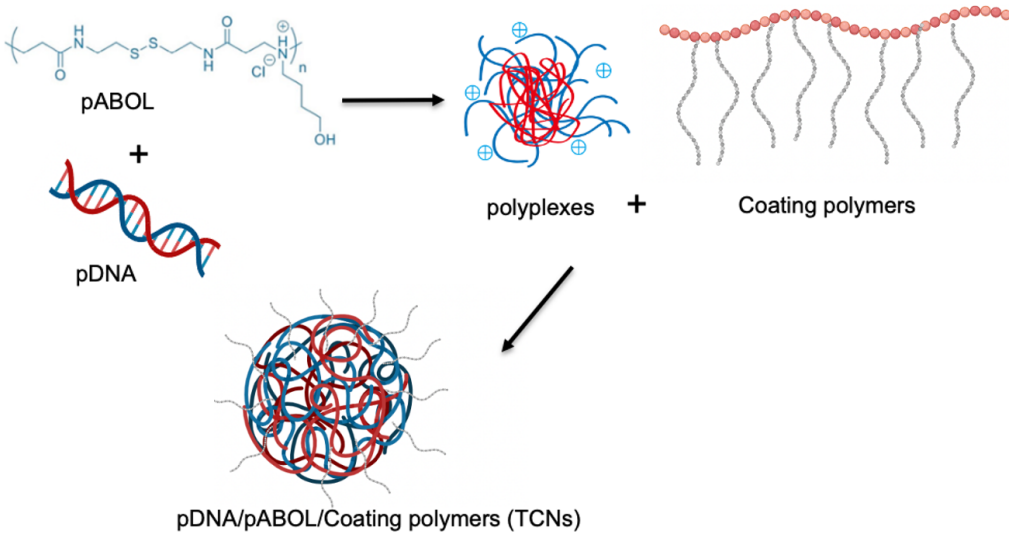
$$\frac{[N]^+}{[P]^-} = \frac{\left(\frac{charge}{mass}\right)pABOL \times (mass)\ pABOL}{\left(\frac{charge}{mass}\right)DNA \times (mass)DNA} \qquad \text{Eq. (1)}$$

The mass to charge ratio of pABOL, pDNA and other coating polymers (PGA and PEG-g-PGA) can be found from **Table 1**. According to the results from previous research, at N:P (charge ratio) of 35 (45:1 w/w), nanoparticles formulated with pABOL larger than 5kDa can have sufficient positive surface charge to maintain the stability and cell permeability. (Blakney et al., 2020) In this study, pDNA was added to the bottom of the tube, pABOL was added to the side of tube at volume ratio (pDNA: pABOL) 1:1 and mixed by vortexing for 30 seconds. The mixtures were incubated at room temperature for 15 minutes to allow for polyplex self-assembly before use or analysis.

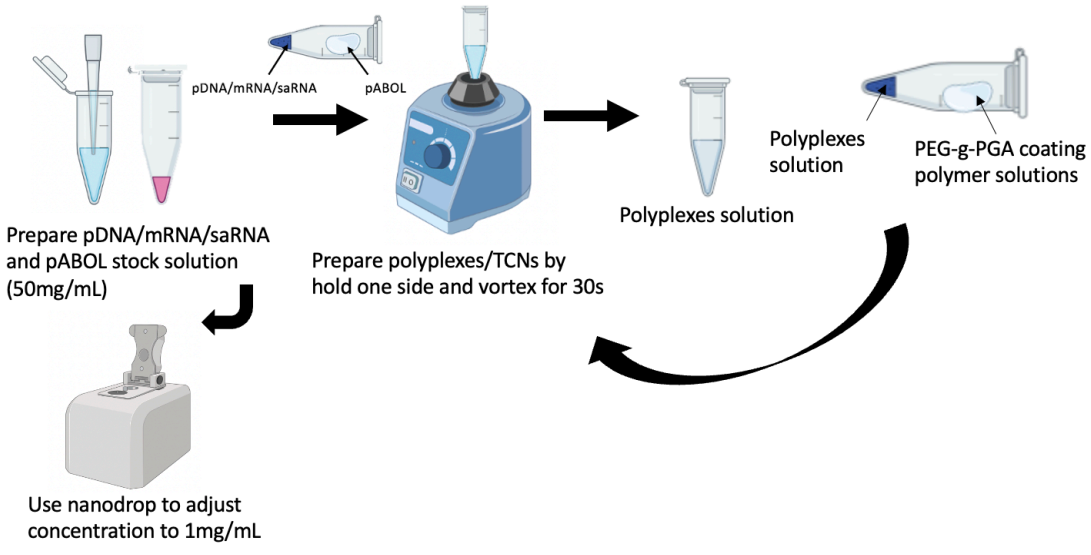
To form TCNs, polyplex solution was added at the bottom of the tube and PGA, or PEG-g-PGA was added to the side of tube at various C (molarity of carboxylic acid charge): N charge ratios (0.1, 0.5, 1 and 5). Polyplexes and coating polymers were mixed at volume ratio = 1:2 by vortexing for 30 seconds and incubated at room temperature for 15 minutes before further characterization.

Electrolyte group	Material name	Net MW (g/mol)	Charged MW (g/mol)	Net polymer mass/charge	Monomer mass/charge
Phosphate (P)	pDNA	3400000	3400000	303.7	303.7
Amine (N)	pABOL	8000	8000	386	396
Carboxylate (C)	15kPGA	15000	15000	150	150
Carboxylate (C)	2.5% PEG-PGA	27800	12513	320	128
Carboxylate (C)	5% PEG-PGA	42800	12126	455	128
Carboxylate (C)	10% PEG-PGA	72800	11352	827	128

Table 1. Molecular weight (MW), charged MW, net polymer mass/charge, monomer mass/ charge of pDNA, pABOL and different coating polymers (PGA, PEG-g-PGA).



Scheme 2. Formulation of self-assemble nucleic acids(pDNA)/pABOL/PGA or PEG-g-PGA TCNs. (pABOL blue; DNA dark red; PGA light red; PEG grey)



Scheme 3. Illustration of how to prepare polyplexes and TCNs

2.5 Characterization of polyplexes and ternary complex nanoparticles

2.5.1 Size and Zeta potential

The physical characteristics of polyplexes and TCNs including hydrodynamic diameter, polydispersity index (PDI), and zeta potential were performed on Zetasizer Nanoseries instrument (Malvern) at 25 °C. To characterize size and PDI of polyplexes and TCNs, the nanoparticle solutions were diluted 100-fold in 20 mM Bis Tris buffer (pH 6.5) or 20 mM HEPES buffer (pH 7.0 and 7.4). To determine zeta potential, samples were diluted 40-fold into 10 mM NaCl solution. All measurements were repeated three times and reported as the mean +/- standard deviation (SD).

2.5.2 Polyplex and TCN DNA condensation

Gel electrophoresis was performed to investigate PP and TCN pDNA condensation. Gels were cast after melting 0.5 g agarose in 100 mL 1X tris acetate EDTA (TAE) buffer with 5 µL 10000X GelRed or SYBR gold. PP and TCN were diluted to obtain 0.34 µg pDNA in 20 µL, mixed with 2 µL loading buffer, and loaded onto 0.5% agarose gel for unpackaging assay. Electrophoresis was carried out at a voltage of 110 V for 40 minutes in 1X TAE buffer and bands were visualized by BioSpectrum imager.

2.5.3 Heparin unpackaging assay

It should be noted that PEG-g-PGA synthesized in aqueous conditions was only used for heparin unpackaging assay due to the limitation of time on optimizing PEG-PGA conjugation reaction. A heparin unpackaging assay was conducted to evaluate whether TCNs improve or worsen polyplexes pDNA packaging. Heparin sodium salt (1000 µg/mL) was diluted to 100, 200, 500 µg/mL in 1mL Bis Tris buffer. Polyplexes and TCNs were distributed to 0.2 mL PCR tubes (0.66 µg pDNA) and incubated with heparin dilutions at 37 °C for 60 minutes. Gel electrophoresis was performed to qualitatively determine the unpackaging of polyplexes and TCNs, and Quant-iT PicoGreen reagent was used to quantify the extent of polyplex and TCN unpackaging. An aqueous working solution of PicoGreen was prepared by making a 200-fold dilution of the concentrated DMSO solution in 1X Tris-EDTA (TE) buffer. Polyplexes and TCNs solution were diluted to 1 ng/µL pDNA. Polyplexes or TCNs diluted with different concentrations of heparin were incubated with an equal volume of PicoGreen at room temperature for 10 minutes, then sample fluorescence was measured by a microplate reader at standard fluorescein wavelength (excitation at 480 nm and emission at 520 nm). Naked pDNA was employed as a standard curve and percent unpackaging was calculated by comparing fluorescent signal of each sample to that of fully unpackaged polyplexes.

2.5.4 DNase I Protection Assay

Similarly, pDNA fluorescence was quantified after challenge with DNase I to stringently evaluate formulation pDNA protection. Polyplexes and TCNs at different N/P ratios and C/N/P ratios were incubated for 30 minutes at 37 °C with DNase I in 10X DNase reaction buffer. DNase I was then inactivated by adding EDTA and incubated for 10 minutes at 65 °C. The amount of DNase I and EDTA added to the system depending on the amount of pDNA in the solution and according to the protocol provided by manufacturers. Heparin (1 μg/μL) was added 1:1 v/v to release pDNA, and pDNA was subsequently stained with PicoGreen through incubation at room temperature for 10 minutes. The pDNA release was assessed by fluorescence quantification at emission of 520 nm and excitation of 480 nm using a fluorescence microplate reader. Naked DNA +/- DNase treatment was used as controls.

2.5.5 Size-stability

The stability of polyplexes and TCNs was characterized as a function of hydrodynamic diameter over a 4-hour incubation at 37 °C using a DynaPro DLS Plate Reader (Wyatt Technology). Polyplexes and TCNs solutions were diluted 5-fold in Bis Tris buffer (pH6.5), PBS buffer (pH7.4) and serum containing media (DMEM+10%FBS) in proprietary cycloolefin 384-well plate (Aurora Microplates). The size of nanoparticles in each well was detected every 30 minutes for 4 hours at 37 °C.

2.6 In vitro transfection

HEK293T cells were seeded in a 48-well plate at 75,000 cells per well and incubated in complete medium (DMEM + 10% FBS) for 24 hours before transfection. After one day, complete medium was replaced with serum-free Opti-MEM and polyplex or TCN solutions containing 1ug of GFP pDNA was added to each well. After 4-hour incubation at 37 °C, polyplexes and TCN solutions were removed, and complete medium was added to wells again. After 20 hours further incubation, an epifluorescence microscope was used to qualitatively check transfection efficiency.

3 Results and Discussion

3.1 Polyplexes and TCNs Optimization

3.1.1 Buffer selection

Knowing that pH can affect polyplex's size and zeta potential, several buffers were evaluated with varying pH to optimize polyplex and TCN formulations. To bind the negatively charged phosphate backbone of pDNA, polymers should be positively charged in the buffer pH range. The pKa of pABOL is about 6.9, which means that at pH = 6.9, 50% of pABOL tertiary amine groups will be protonated. We hypothesized that at

lower pH (more positively charged amines), pDNA would be more fully condensed and achieve smaller size with more positive zeta potential. In addition, a more positively charged polyplex would be more stable in solution because of electrostatic repulsion between particles.(Pack et al., 2005) However, because PGA's pKa ~ 5 and PGA must be deprotonated to coat polyplexes, the chosen buffer's pH should be in the range of 5-7 to ensure that pABOL is protonated and PGA is deprotonated.

Oddly, the majority of research with pABOL uses pH 7.4 HEPES buffer, at which pH only ~15% of pABOL amines will be positively charged. For example, saRNA formulation with pABOL was performed in 20 mM HEPES buffer with 5% (w/w) glucose at pH 7.4 (Blakney et al., 2021) or in 10mM HEPES buffer at pH 7.4.(Démoulins et al., 2016). However, lower pH buffers like sodium acetate buffer are also commonly used to make polyplexes. For example, the Stephan group manufactured TCNs by mixing pDNA, polycation, and PGA in 25 mM sodium acetate buffer at pH 5.2 and the resulting particle size was around 155nm. (Smith et al., 2017)

In this study, three different buffer systems and four pH's were chosen to evaluate the impact of buffer pH on particle size and stability: pH 5.2 sodium acetate, pH 6.5 bis tris, and pH 7.0/7.4 HEPES. As shown in **Figure 1**, it is clear that pDNA, mRNA (gift from Dr. Fabio Fisher), and saRNA (gift from Prof. Robin Shattock) pABOL polyplexes are very stable in pH 6.5 Bis Tris buffer and pH 7.0 HEPES buffer without any aggregation within 4 hours in room temperature. However, these polyplexes were not stable at pH 7.4; the size of saRNA and mRNA polyplex was almost twice of the respective polyplex at pH 6.5 and pH 7.0. Moreover, for mRNA polyplexes aggregated within 4 hours.

Due to the relative stability of pDNA, further analysis of polyplexes and TCNs was conducted with pDNA instead of mRNA or saRNA (although future work will return to RNA). The size distribution of pDNA/pABOL polyplexes formulated in different buffer systems can be found in **Figure 2**. pDNA/pABOL polyplexes made in pH 6.5 Bis Tris buffer has narrow size distribution (PDI=0.161 ± 0.03), and the size was much smaller than those made in acetate and HEPES buffer. However, the results of two experiments were contradictory for pH 7.4 HEPES buffer, which could be related to the concentration difference of polyplexes solution tested by two instruments. Polyplexes were diluted 5-fold for stability test on the DynaPro DLS Plate Reader in an Aurora 384-well plate, whereas polyplexes were diluted 100-fold for analysis on the Zetasizer in a PMMA cuvette. In a high particle concentration environment, the electrostatic repulsion may be stronger than in the low concentration environment, which may lead to smaller size

detected by DLS. Nevertheless, polyplexes prepared in pH 7.4 HEPES buffer were not reproducible, and pH 6.5 Bis Tris buffer was chosen to formulate polyplexes and TCNs for further experiments.

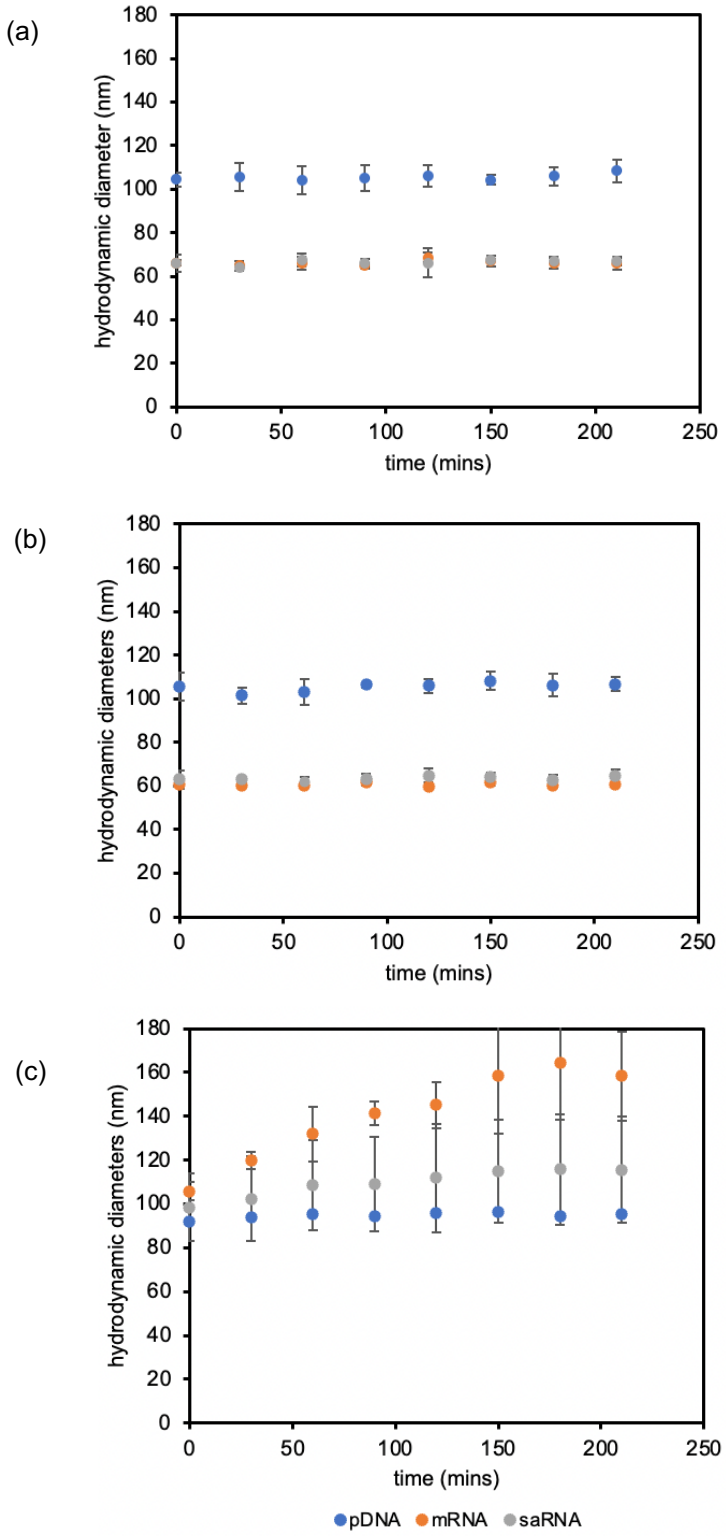


Figure 1. pDNA /mRNA/saRNA pABOL polyplex stability in (a) 20 mM Bis Tris buffer (pH 6.5), (b) 20 mM HEPES buffer (pH 7.0); (c) HEPES buffer (pH 7.4) (N = 3 technical replicates plotted as mean ± SD)

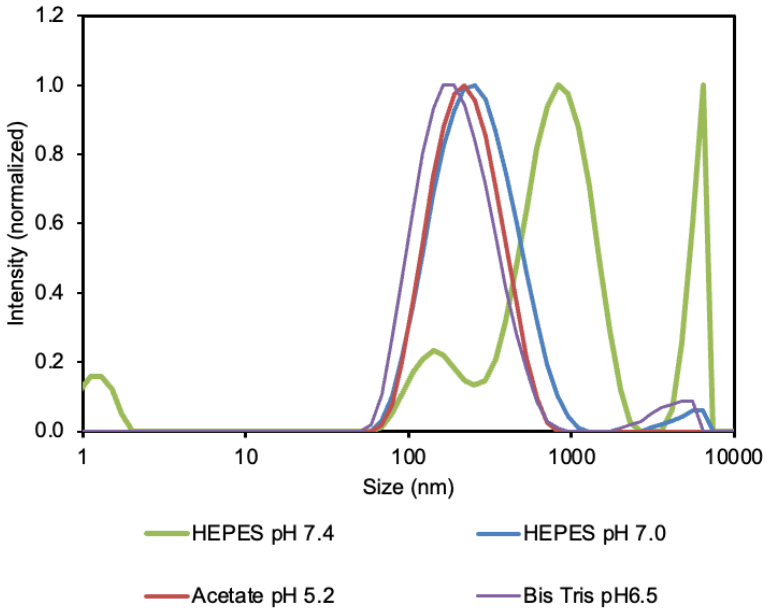


Figure 2. The size distribution of pDNA/pABOL polyplex synthesized in different buffer with various pH at room temperature detected by DLS.

3.1.2 PGA selection

We hypothesized that, analogous to polyplex self-assembly, ternary complex self-assembly must occur very rapidly and homogenously in order to avoid aggregation and minimize particle size. Because the molecular weight of polyelectrolytes influences self-assembly kinetics, we compared the size and zeta potential of TCNs formed with both low (3-5 kDa) and moderate (15 kDa) MW PGA at various C:N charge ratios. **Figure 3** shows that the addition of LMW or MMW PGA similarly decreased PP zeta potential from +30 to -25 mV at C:N = 2.4. For all TCNs, the zeta potential was lower than polyplexes at C:N > 0.6. However, for MMW PGA TCNs, the decrease of zeta potential was accompanied with an increase in particle size, indicating that the addition of MMW PGA induces aggregation at intermediate charge ratios. At C:N = 0.6 and 1.8, TCNs formed aggregates due to weakened electrostatic repulsion during assembly; in this situation, one PGA may connect several polyplexes during TCN self-assembly. At C:N=2.1 and 2.4, electrostatic repulsion during TCN assembly was enhanced by the excess coating polymer, helping to condense individual polyplexes and achieve smaller size. TCN formulations were also examined with a gel retardation assay. From **Figure 3**, naked pDNA was detected as 3 bands (with varying supercoiling) on 0.5% agarose gel. As bands of pDNA were not detected in any TCN lanes, adding a large amount of PGA as coating polymer to polyplex does not lead to dissociation of polyplexes. In other words, the addition of PGA did not affect the binding between pABOL and pDNA.

At sufficiently high C:N, the size and zeta potential of LMW PGA or MMW PGA TCNs were similar. However, MMW PGA provided more reproducible results compared with LMW PGA, and would provide more flexibility for further PEG conjugation. LMW PGA has a degree of polymerization (DP) of 20-25 glutamates, while MMW PGA DP = 100, enabling a wider range of grafting densities to be explored in later experiments. Thus, MMW PGA was chosen for further experiments due to its satisfactory TCN properties and ease of conjugation.

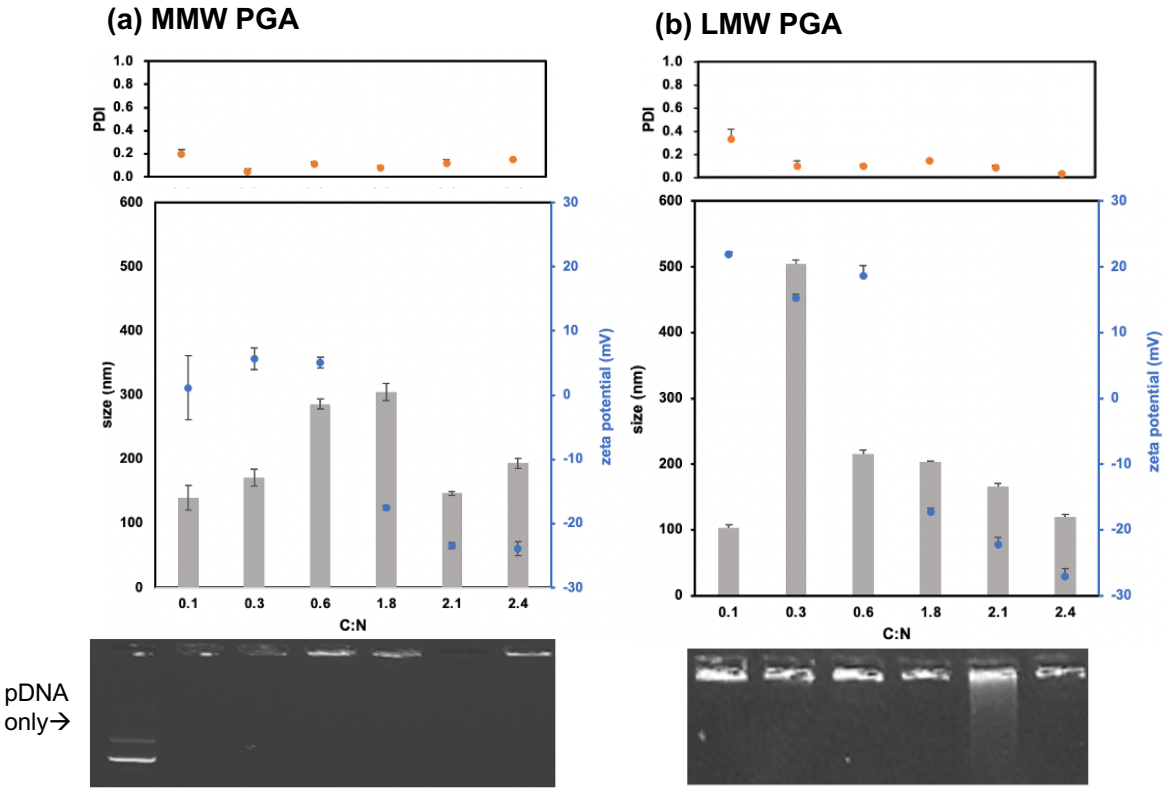


Figure 3. Hydrodynamic diameter, zeta potentials and gel electrophoresis of TCN formulated with (a) MMW PGA or (b) LMW PGA at various C:N charge ratios.

3.2 Characterization of PGA-g-PEG

Two different conjugation methods were applied to synthesize PEG-g-PGA with different percentages of PEG. One method employed purely aqueous conditions (BBS pH 8.0) and the other conducted part of the reaction in DMSO to avoid premature hydrolysis. Aqueous Gel Permeation Chromatography (GPC) was used to determine the purity and molecular weight distribution (dispersity, ~~*Đ*~~) of the conjugated polymers. From **Figure 4**, it is clear that unconjugated PEG still remained after dialysis in both aqueous and part-organic reactions. These results indicate that the conjugation reaction was not 100% efficient and that a 10 kDa dialysis bag could not fully remove unreacted 5 kDa PEG.

Table 2 further shows that the distribution of conjugate molecular weights (*Đ*) is higher in aqueous conditions compared with part-DMSO conditions. However, from **Figure 4**, there are obvious shifts comparing PEG-g-PGA with PGA only, indicating that some PEG was conjugated to PGA by EDC/NHS reaction. The aim of this study is to see the effects of PEGylated PGA on stability and transfection efficiency of TCNs. In this case, these conjugated polymers could still be used for further experiment although some free PEG was present in the system. Ongoing work is focused on refining the conjugation and purification of these polymers.

mPEG5k-NH_2 was covalently coupled to the PGA backbone through an amide bridge with EDC and NHS. The grafting percentage of PEG was calculated by ^{1}H-NMR (**Figure 5**). However, it is not possible to determine the true grafting percentage from NMR results because of the presence of unreacted PEG (**Figure 4**). Because free PEG is not expected to influence TCN stability and transfection efficiency, we proceeded with these materials and used the obtained grafting results from NMR for later formulation calculations.

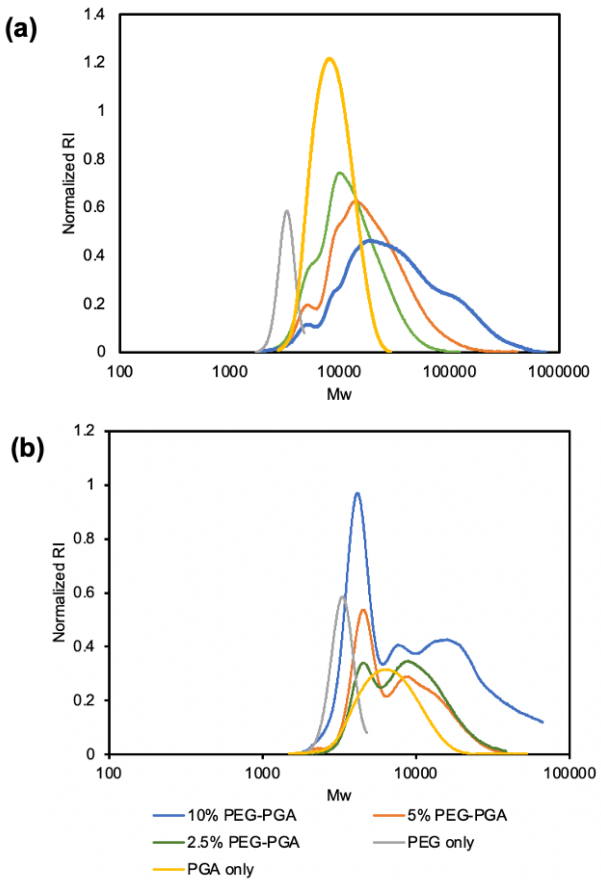


Figure 4. GPC chromatograms analysis: (a) PEG grafted PGA in BBS buffer (aqueous condition); (b) PEG grafted PGA in part of DMSO (organic condition)

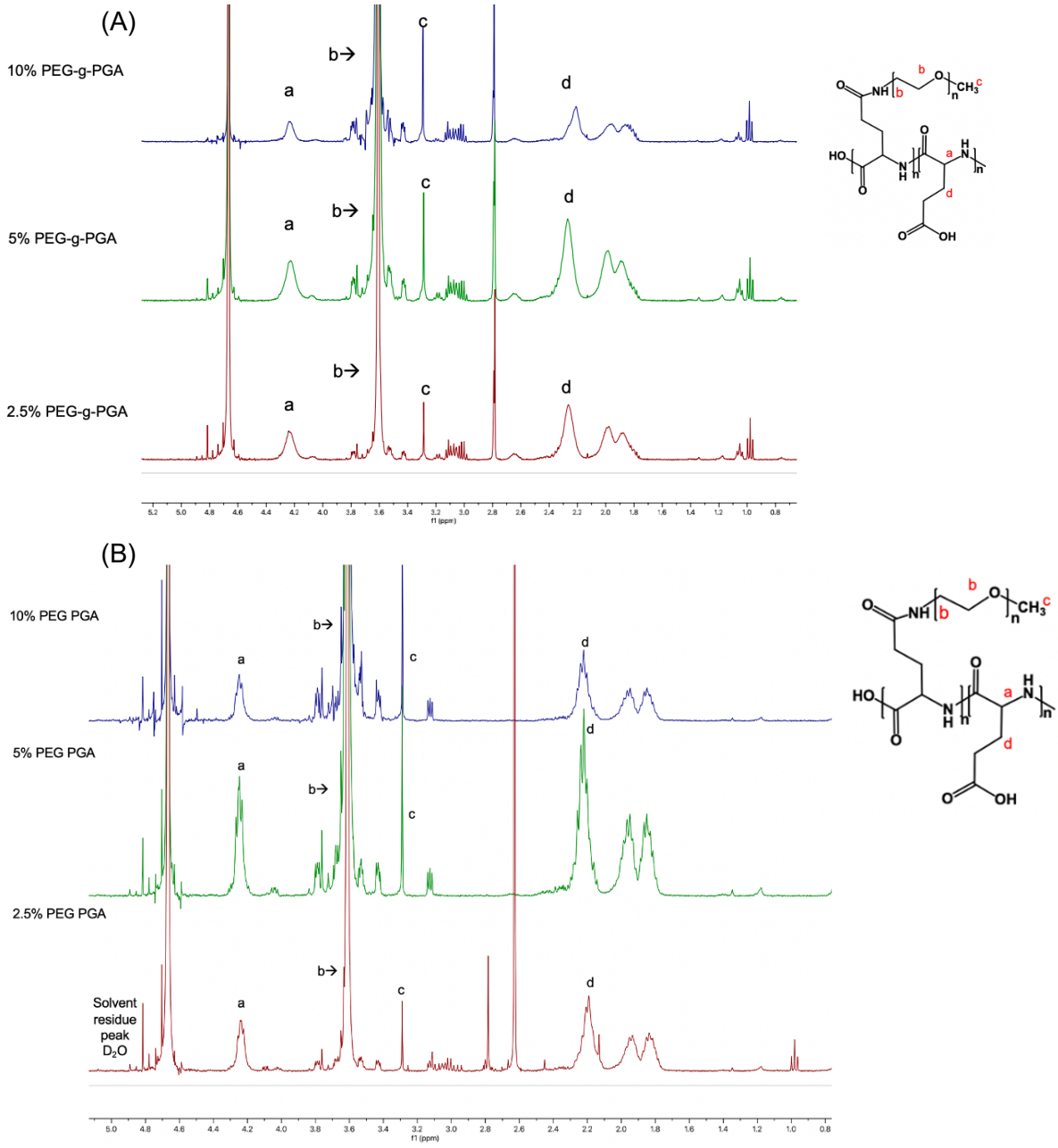


Figure 5. NMR spectra of mPEG5k-NH2 grafting alpha PGA: (A) ^{1}H NMR spectrum of PEG-g-PGA conjugation in aqueous condition; (B) ^{1}H NMR spectrum of PEG-g-PGA conjugation in part DMSO condition. (Note: PGA-g-PEG ^{1}H NMR (400 MHz, D2O) spectra, peak d at 2.2 ppm (2H,CH2-CH2-CO), peak c at 3.23 ppm (PEG: O-CH3), peak b at 3.55 pm (PEG:CH2-CH2-O); peak a at 4.16 ppm (PGA: NH-CH-CO).)

Condition	Polyanion	NMR	GPC	
		Mn (Da)	Mn (Da)	Đ
Aqueous	2.5% PEG-g-PGA	40000	9988	1.43
	5% PEG-g-PGA	55000	13005	1.71
	10% PEG-g-PGA	115000	17956	2.58
DMSO	2.5% PEG-g-PGA	27800	10317	1.15
	5% PEG-g-PGA	42800	10075	1.21
	10% PEG-g-PGA	72800	12713	1.40

Table 2. Characterization of PEG-g-PGA conjugation via NMR and GPC. (Mn calculated from NMR spectroscopy is based on assumption that all PEG present is conjugated. The difference of Mn calculated by NMR and GPC is due to unremoved PEG in the system)

3.3 Characterization of TCN

3.3.1 Size and zeta potential

The particle size and zeta potential of PGA and PEG-g-PGA TCNs were then characterized. The results (**Figure 6**) indicate successful TCN formulation. Polyplexes in 20 mM pH 6.5 Bis Tris buffer have a diameter of 69.01 ± 2.3 nm and zeta potential of 28.6 ± 0.8 mV. After coating with different PEG-g-PGA at C:N=0.1 to 5, the size of TCN was around or above 200 nm, nearly three times that of polyplexes. It is noted that the PEG only adds a few nanometers to the polyplexes' diameter.(Guo et al., 2011) In this case, what has been observed is polyplex aggregation during TCN assembly process. Comparing the size of TCN (PGA) only and TCN (PEG-g-PGA) at the same C:N ratio, the size of TCN (PGA) only was much higher than the size of TCN (PEG-g-PGA). Also, polyplex aggregation is suppressed at high PEG ratios, which can be observed from **Figure 7(a).** This phenomenon may be caused by enhanced steric shielding between particles due to PEG on the particle surface.

As expected, the zeta potential of TCNs changed from positively charged to negatively charged as the C/N ratio of PGA or PEG-g-PGA to pABOL increased, which confirmed that TCNs were successfully synthesized from **Figure 7 (b)**. The zeta potential of TCN (PGA) at C:N=5 is much lower than all TCN (PEG-g-PGA) at C:N=5, which indicates that PEG dampens the magnitude of nanoparticle surface charge as reported in literature(Guo et al., 2011; Xia et al., 2016). However, the relationship between PEG density and surface charge of TCNs is not obvious from these results.

3.3.2 Gel retardation assay

The capacity of the polyanion to disassociate the polyplexes were studied by a gel electrophoresis assay. **Figure 6** shows the gel electrophoresis assay images of TCN (PGA) and TCN (PEG-g-PGA) at C:N = 0.1, 0.5, 1 and 5. At C:N $\geq$ 0.5, a free DNA band was detected for TCN (PEG-g-PGA**)** but not for TCN (PGA), indicating that PEG-g-PGA caused DNA dissociation from the polyplex by competitive binding with pABOL, which may result in a weakening of the interaction between pDNA and pABOL. However, adding bare PGA did not cause any loosening of the polyplex. Moreover, this PEG-dependent unpackaging phenomenon has not also been observed for pDNA/PEI/PEG-g-PGA TCNs in the literature. (Xia et al., 2016)

This disassociation phenomenon may be due to 1) the relatively low molecular weight and charge density of 8 kDa pABOL compared to 25kDa PEI, and/or 2) hydrophilic solubilization driven by PEG dominating the electrostatic force between PGA and pABOL. Past work has shown that the loosening capacity of polyanions depends on the pKa of the anionic functional group as well as the flexibility of the polyanion chain, where lower anion pKa and more flexibility led to more pDNA/polyplexes loosening.(Wang et al., 2010) Thus, the enhanced flexibility of PEG-g-PGA relative to PGA may also contribute to the observed results. Moreover, since TAE buffer with pH 8.5 was used as running buffer for gel electrophoresis, the TAE buffer's pH may affect pABOL and coating polymers on pDNA condensation. At pH 8.5, polyanions (PEG-g-PGA) were deprotonated more than at pH 6.5 bis tris buffer and pABOL was less protonated, which biases against pDNA condensation and leads to non-physiological dissociation of polyplexes. Future work will compare TCN packaging with gel electrophoresis conducted at pH 7.4 to evaluate differences in TCN assembly more realistically.

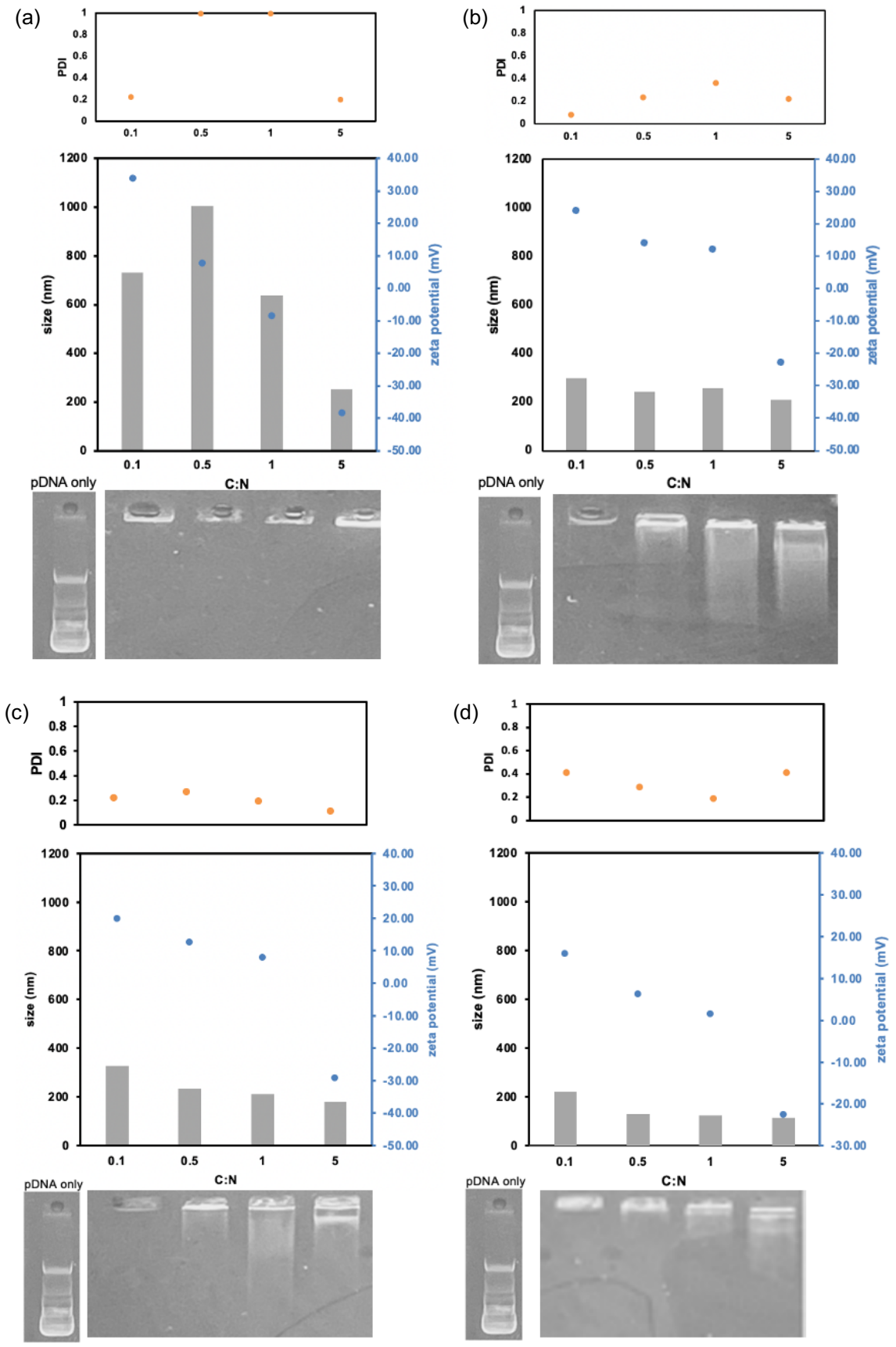


Figure 6. Size, PDI, zeta potential and gel electrophoresis results of (a) PGA (b) 2.5% PEG-g-PGA (c) 5% PEG-g-PGA (d)10%PEG-g-PGA as coating polymer

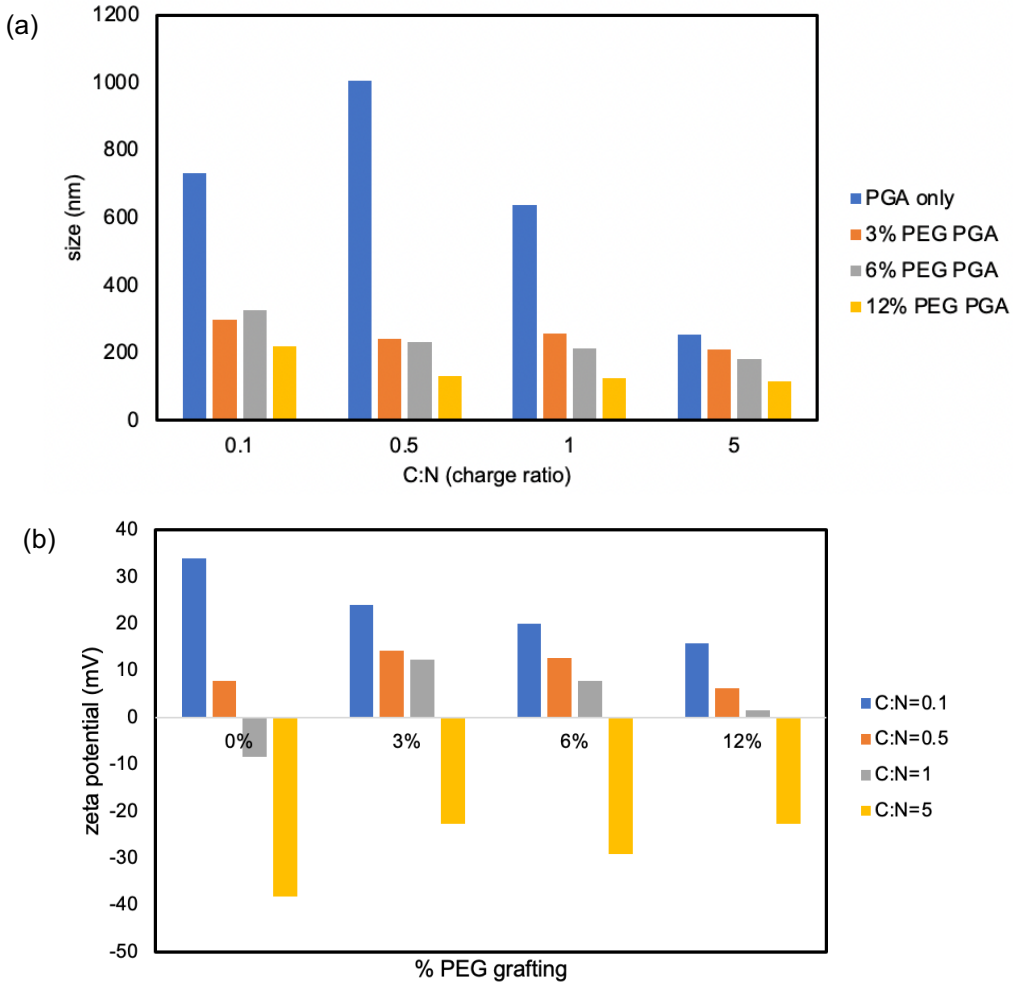


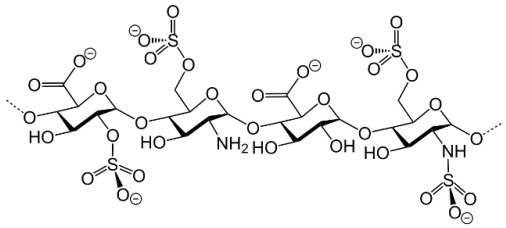
Figure 7. Comparison size(a) and zeta potentials (b) of TCN at various C:N with different coating polymers.

3.3.3 Heparin unpackaging experiment

To simulate an extremely challenging *in vivo* environment that could unpackage TCN (PEG-g-PGA), we assessed the release of pDNA from polyplexes and TCNs (PEG-g-PGA: synthesized in aqueous conditions) in the presence of extremely high concentrations of heparin. There are repeating sulfo groups in the heparin which can be seen from **Scheme 4**. Heparin is known to have the highest negative charge density of any macromolecule and competes with pDNA for binding to pABOL. In this study, we put polyplexes and TCNs in extreme heparin conditions compared with 1 μg/mL heparin found in physiological conditions to investigate differences in unpackaging resistance. **Figure 8** qualitatively shows that pDNA was released from polyplex and TCN with increasing heparin concentration from 50 μg/mL to 250 μg/mL. There was no discernable

difference between formulations except perhaps at the lowest concentration tested, where less pDNA was displaced from highly PEGylated TCNs.

The effect of heparin on the stability of complexes was also quantitatively evaluated at lower concentrations as a change in fluorescence intensity obtained with PicoGreen. Because PicoGreen binding to pDNA is decreased in the presence of PEG (**Figure 9a**), we present data normalized to the fluorescent signal without heparin for each sample (**Figure 9b**). It can be seen from **Figure 9a** that in the absence of heparin, some pDNA is still detected by fluorescence, which means that PicoGreen still has accessibility to pDNA even in the polyplex and TCN. After normalization to the baseline signal observed without heparin (**Figure 9b**), the heparin concentration at which half the maximum polyplex and TCN unpackaging observed is the same for polyplexes, 8%PEG-PGA TCN, and 15% PEG-PGA TCN, indicating that PEGylation does not restrict heparin access to polyplexes. To further optimize TCN *in vivo* stability, additional hydrophobic moieties could be added to PGA to combat heparin's electrostatic forces.



Scheme 4: Structure of heparin

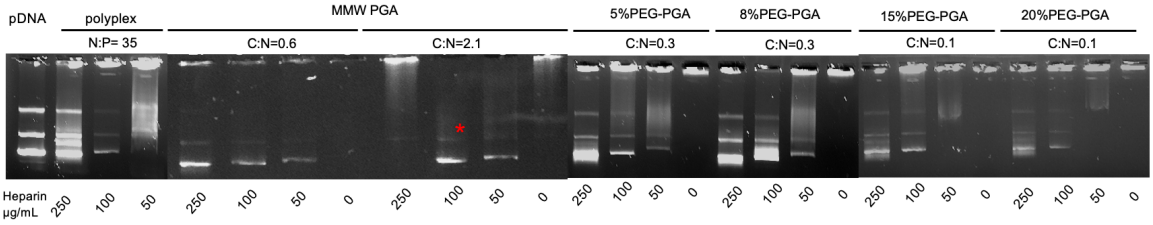


Figure 8. Gel electrophoresis results of PP and TCN unpackaging by different concentration of heparin. (*unexpected result due to no heparin was added)

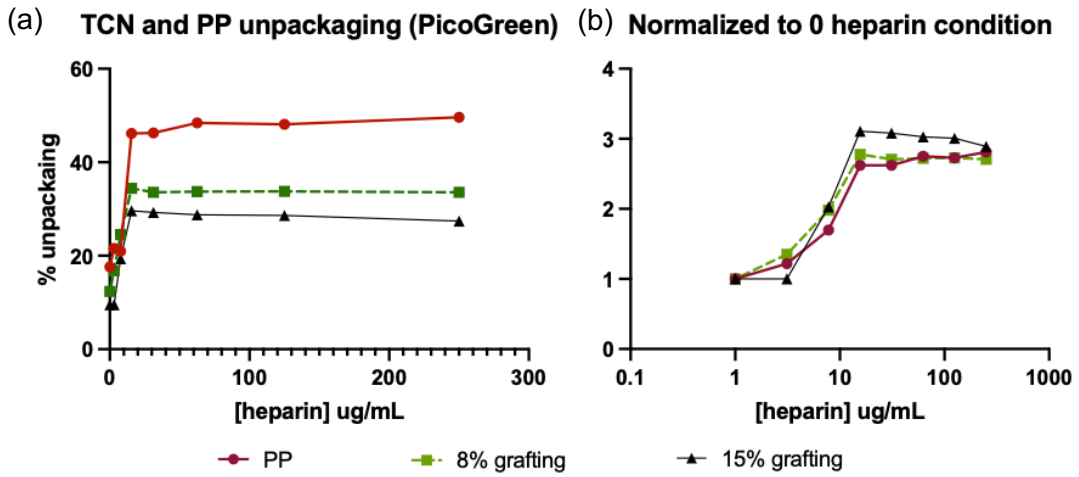


Figure 9. PP and TCN unpackaging in different concentrations of heparin. **(a)** % unpackaging of PP and TCN calculated by [(Sample fluorescence) -(Fluorescence at 0 μg/mL heparin)]/(Fluorescence at 250 μg/mL heparin) **(b)** data normalized to the fluorescent signal without heparin

3.3.4 DNase I Protection assay

Protecting pDNA from degradation by nuclease is necessary for gene delivery *in vivo*, and DNase I is known to be the major nuclease found in serum. Therefore, to investigate the ability of pABOL, TCN (PGA), and TCN (PEG-g-PGA) to protect pDNA from degradation, DNase I protection assay was conducted by gel retardation and fluorescence quantification assay. From **Figure 10b**, naked pDNA was completely degraded by DNase I with or without 10X reaction buffer within 30 minutes at 37 ℃. In contrast, **Figure 10a** showed that N:P = 35 pABOL polyplexes had 52% pDNA remaining and was higher than N:P=12 pABOL polyplex and PEI polyplex (positive control). However, the result was contradictory to other literatures which showed good protection of PEI (Özgel & Akbuğa, 2006). Probably because of the ongoing degradation by partially active DNase I after PEI polyplex unpackaging. Moreover, from polyplex characterization results, N:P=35 pABOL polyplex's zeta potential is about +28mV, indicating that pDNA was condensed by pABOL completely; this result was further confirmed by gel retardation assay showing no pDNA band detected from polyplexes. However, from fluorescence quantification assay (**Figure 10a**), there was about 30% pDNA detected in pABOL N:P=35 polyplex without DNase I treatment, indicating that PicoGreen may have access to pDNA in the polyplex or pABOL polyplex is loosely packaged. From **Figure 11**, there was 36.8% pDNA remaining from TCNs (12%PEG-g-PGA) after DNase I treatment, which is higher than other TCNs and polyplexes, indicating that PEG might restrict the approach of nuclease to pDNA. However, there was only small amount of

pDNA remain after DNase I and heparin treatment, which means that after adding heparin into the system to release pDNA, pDNA was inadvertently degraded immediately by the partially inactivated DNase in the system. Thus, further work will be needed to refine this assay to ensure full DNase I inactivation.

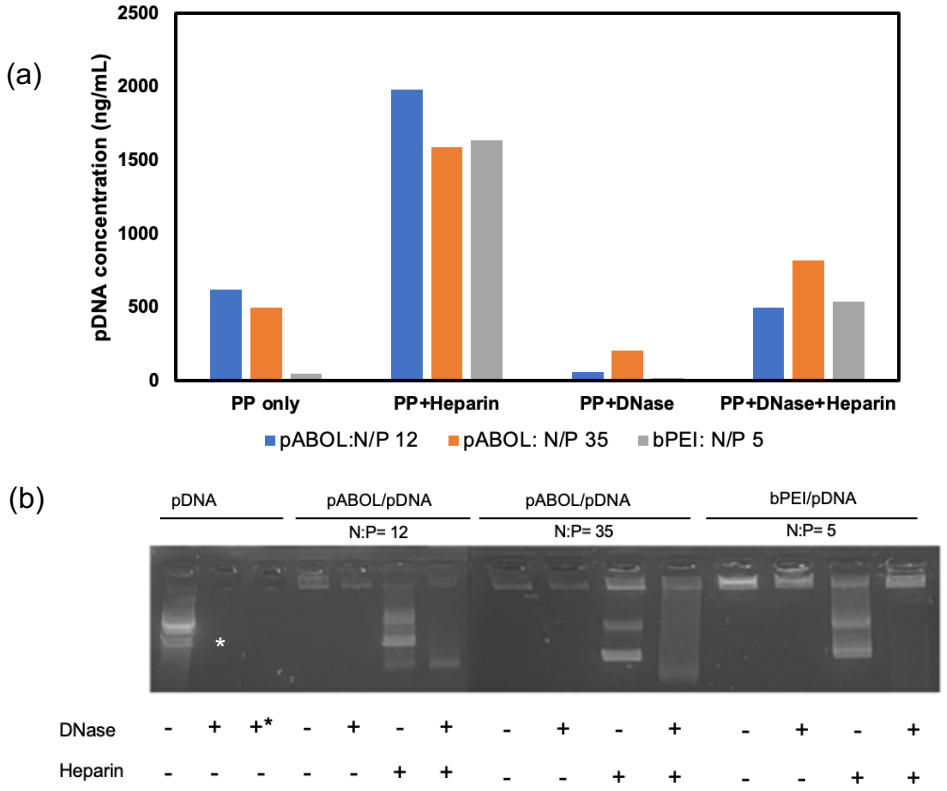


Figure 10. DNase protection of bPEI and pABOL at different N/P (charge ratio) detected by **(a)** fluorescence quantification and confirmed by **(b)** gel electrophoresis**.** (* pDNA treated with DNase without 10X reaction buffer, other DNase treatments are all with 10X reaction buffer)

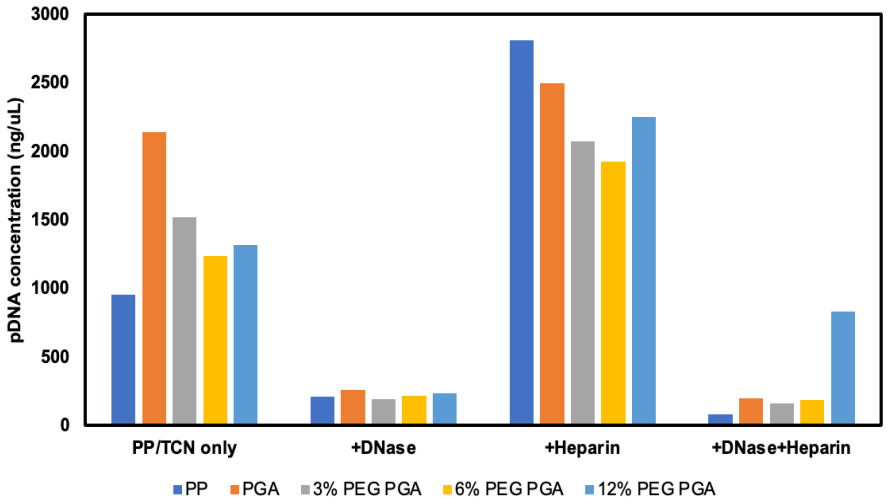


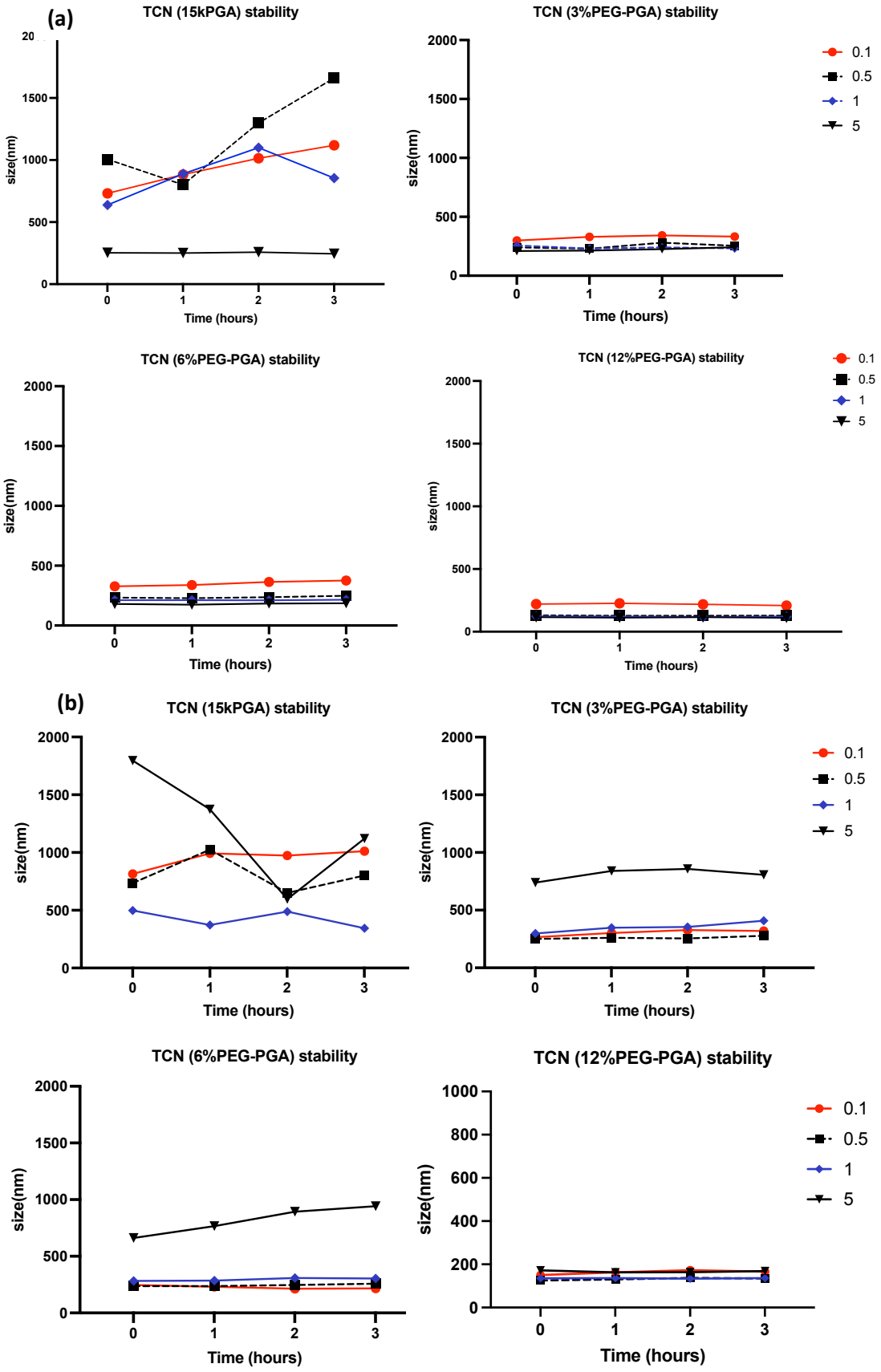
Figure 11. pDNA concentration of pABOL/pDNA polyplexes at N:P=35, TCN (PGA) and TCN (PEG-g-PGA) at C:N=5 after Heparin and DNase I treatment

3.3.5 Size stability in various media

Maintaining small particle size in salt- and serum-rich physiological environments is essential for efficient transfection *in vivo* and for LN drainage of TCNs. Thus, we next investigated the stability of TCNs in various environments by tracking changes in particle size with DLS over 4 hours at 37 °C. Specifically, we sought to investigate whether PGA or PEG-g-PGA could inhibit polyplex aggregation in pH 7.4 PBS or serum-containing medium. From **Figure 12b,** the size of TCN (PGA) was above 500 nm at all tested C:N ratios indicating the aggregation induced by salt in PBS. Thus, electrostatic screening results in TCN (PGA) instability in saline, just as observed for polyplex alone and other nanoparticles in literature.(Burke & Pun, 2008) Additional forces beyond electrostatics are thus needed for stabilization in the presence of salt, namely, steric shielding via PEG-g-PGA. TCNs (PEG-g-PGA) at C:N=0.1 to 1 achieved smaller size (around 250 nm) relative to TCNs (PGA) at same C:N ratios and were stable in PBS for at least 4 hours. Also, the size of TCN (12%PEG-PGA**)** became about 150 nm, indicating that TCNs became more stable and more condensed by increasing percentage of PEGylation. However, there is almost no difference between stability of TCNs (3 & 6%PEG-PGA) in PBS buffer at various C:N ratios; their actual PEG conjugation percentages are similar, as demonstrated by GPC (**Figure 4b**). Yet this comparison also shows that soluble PEG (there is twice as much in the 6% PEG-PGA formulation vs 3%) does not affect TCN stabilization in PBS.

As expected, polyplexes aggregated in serum. The corresponding size distribution contained multiple peaks instead of one narrow peak in pH 6.5 Bis Tris buffer as shown in **Figure 13**. This aggregation was caused by interaction between positively charged polyplexes and negatively charged serum proteins as shown in **Scheme 5**. In contrast, From **Figure 12c**, TCN (PGA) treated with 10% FBS did not show big aggregation at C:N= 5 in comparison with the size in Bis Tris buffer at C:N = 5. However, aggregations were formed for TCN (PGA) at C:N=0.1 to 1. Although the size of TCN (PGA) was almost constant at C:N=0.1 (**Figure 12c**), the PDI was multimodal leading to the wrong calculation by DLS. From the results of TCN characterization (**Figure 6a**), the zeta potential of TCN (PGA) at C:N=5 is -40 mV so the stability may be achieved via repulsive electric forces between TCNs and serum proteins. Comparing with TCNs (PGA), TCNs (PEG-g-PGA) were stable with constant size around 250 nm at C:N 0.5 to 5 in 10% FBS, indicating that PEGylation can help to stabilize polyplex structure in the presence of serum proteins. Also, it can be seen from **Figure 12c** that TCNs (12%PEG-PGA) were more stable than TCNs (3&6%PEG-PGA) at C:N=0.1, indicating PEG grafting density

affects TCN stability by modifying steric effects and thus decreasing particle and protein interactions.(Mishra, Webster & Davis, 2004)



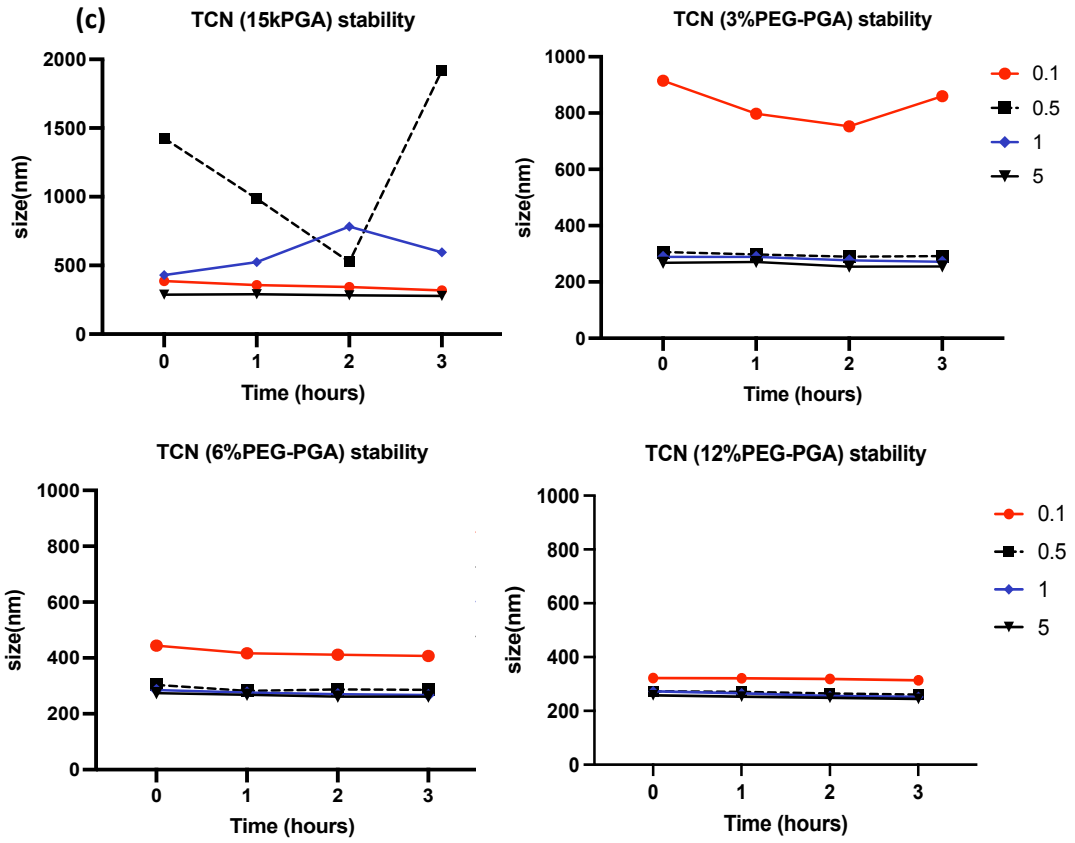


Figure 12. TCN (PGA or PEG-g-PGA) stability characterized by size change every 30 minutes at 37 °C in **(a)** Bis Tris buffer, **(b)** PBS, and **(c)** Complete media.

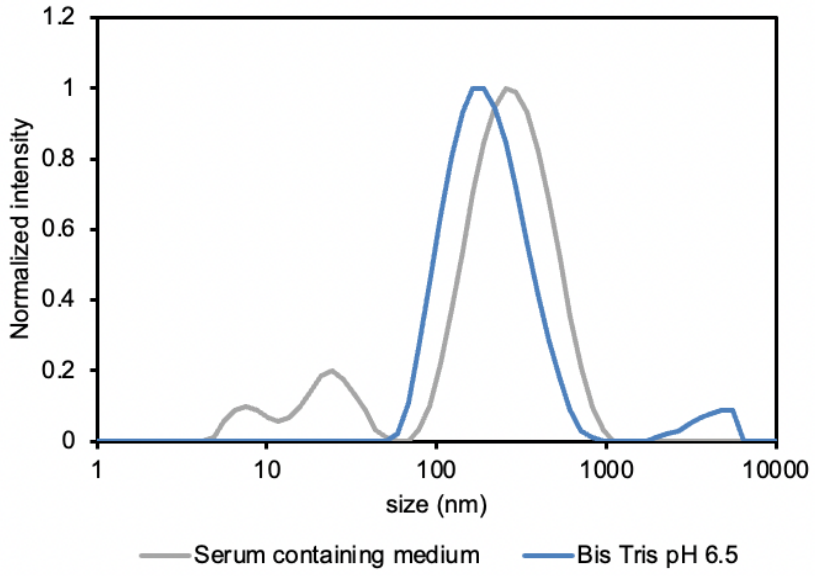
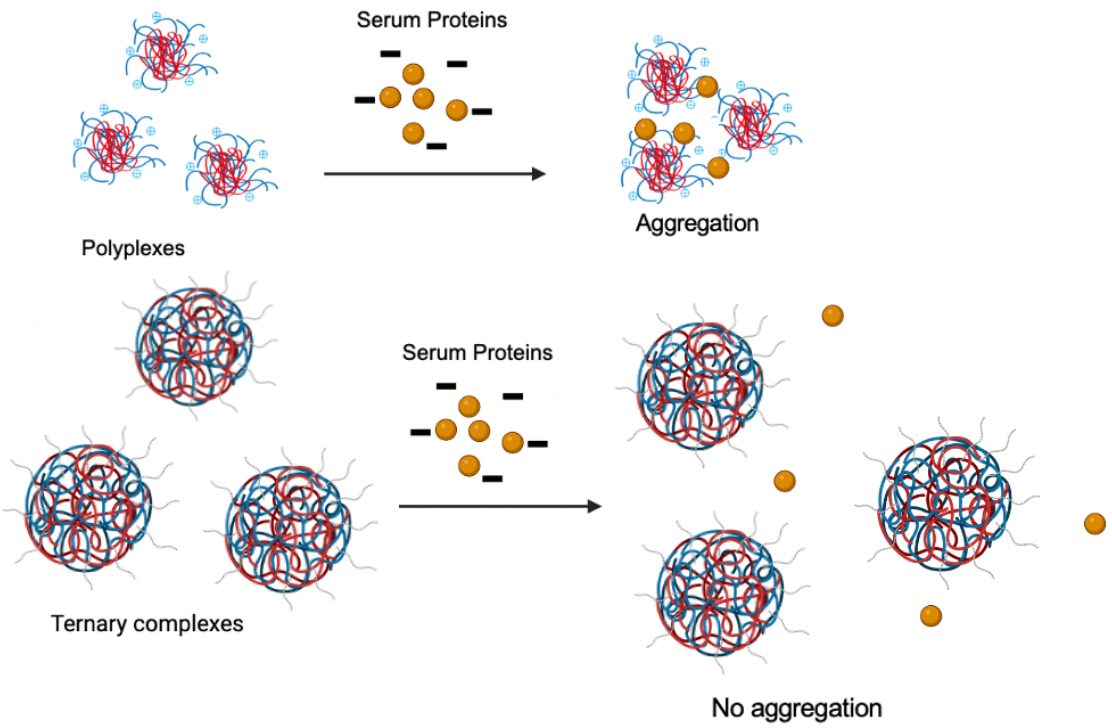


Figure 13. Size distribution of polyplexes in serum containing medium vs. Bis Tris buffer.



Scheme 5. Illustration of serum protein adsorption in complete medium which results in differential stability of polyplexes vs. ternary complexes

3.4 *In vitro* Transfection

To investigate whether coating polymers impact polyplex function, polyplexes, and TCNs were assessed for *in vitro* GFP transfection efficiency in serum-free (Opti-MEM) and serum-containing (DMEM+10% FBS) medium. In this experiment, TCNs were formulated at C:N=5 because of their smaller size and stability in 10% FBS than at other C:P ratios (**Figure 12 c**). As before, PGA and PEG-g-PGAs were compared to figure out whether PEGylation and PEG grafting density would impact transfection efficiency.

It can be observed from **Figure 14** that the pDNA transfection efficiency of TCNs (PGA) at C:N = 5 was higher than that of polyplexes and TCNs (PEG-g-PGA) under serum-free medium. However, this result was contradictory to the stability test (**Figure 12b**), in which TCNs (PGA) aggregated in PBS. Also, negatively charged complexes usually have poor cellular uptake because of the repulsion from the negative charged cellular membrane. In this case, there should be a different mechanism for PGA-coated nanoparticles to enter the cells: 1) Aggregated TCNs (PGA) precipitated on the cell surface and thus increased endocytosis or 2) There is a specific pathway for PGA-coated nanoparticle entering the cell. Kurosaki et al showed that γ-PGA entered the cells via γ-PGA specific receptor-mediated pathway.(Kurosaki et al., 2009) However, for α-PGA, no such pathway has been reported. Unsurprisingly, transfection efficiency in serum-free medium

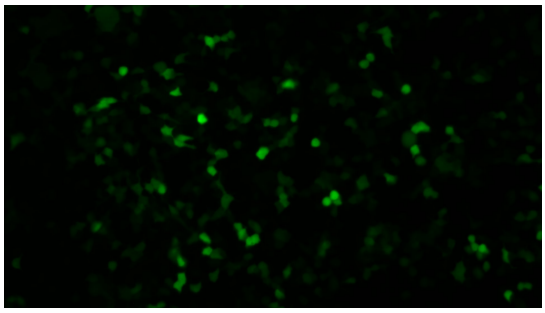
decreased with increasing of percentage of PEG conjugated to PGA. It is known that dense PEGylation inhibits nanoparticle uptake *in vitro*, but also that this not predictive of *in vivo* uptake and transfection.(Suk et al., 2016) Importantly, these results also demonstrate that PEGylation has significantly modified the surface of the polyplexes to decrease non-specific uptake and allows future work to focus on engineering in ligands for cell-type specific uptake.

In contrast, TCNs (PEG-g-PGA) retained more transfection than TCNs (PGA) and polyplexes in 10% FBS, although transfection was very low overall. Positively charged polyplexes had almost no gene transfection efficiency in serum due to the strong absorption to negatively charged serum proteins. By introducing polyanions such PGA or PEG-g-PGA to polyplexes, the nonspecific bonding between serum proteins and complexes decreased, thus increasing gene transfection efficiency. From **Figure 14 b**, the transfection efficiency of TCNs (PEG-g-PGA) were very low but higher than TCNs (PGA). The reason behind this phenomenon is that the steric effects of grafted PEG limits the interaction of TCNs with serum proteins while high density of PEG may reduce cellular uptake of nanoparticles. (Mishra, Webster & Davis, 2004)

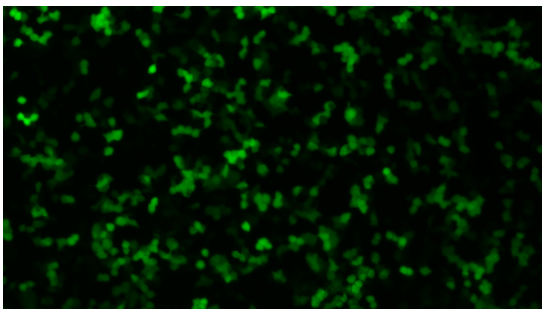
(a) Serum-free medium (Opti-MEM)

Control (cell only)

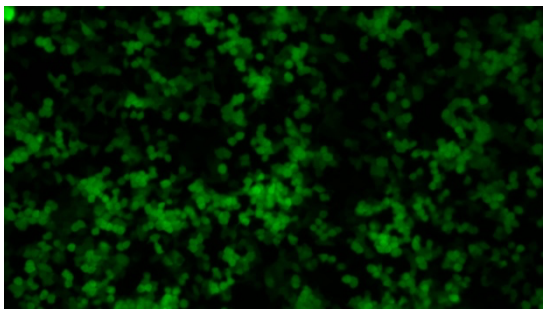
Polyplexes, N/P 35



TCN (PGA) C/N 5



TCN (3% PEG-PGA) C/N 5



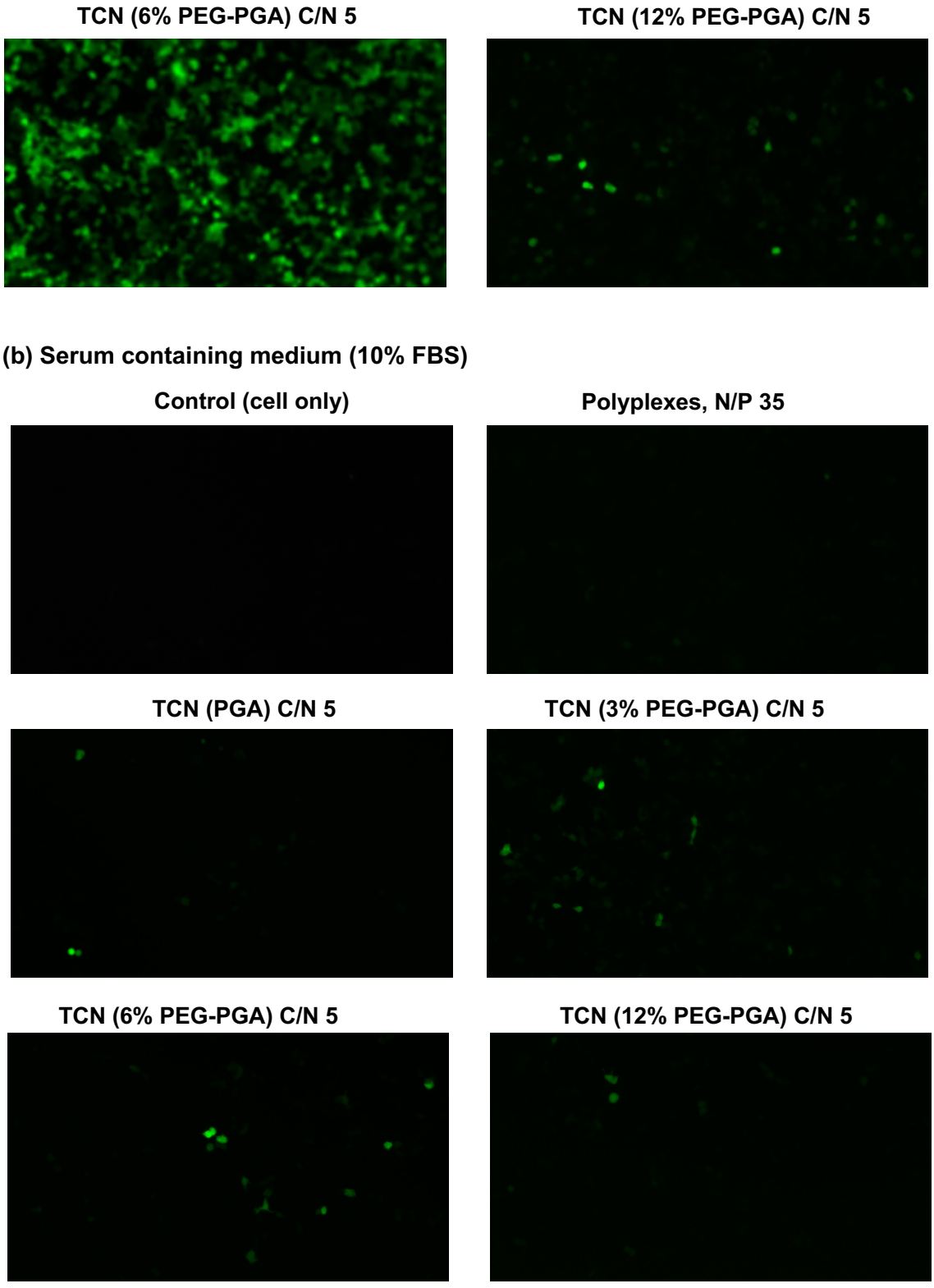


Figure 14. Fluorescence micrographs of polyplexes (pABOL/pDNA N/P 35) and TCN (PGA only: C:N=5; PEG-PGA C:N=5) transfection in HEK 293T.17 cells in **(a)** serum free medium and **(b)** serum containing medium .

4 Limitations and Future works

4.1 Limitations

There are several limitations of this study: 1) Unreacted PEG impurities in PEG-g-PGA samples (identified via GPC) make it impossible to calculate the true PEG grafting percentage, net polymer mass to charge ratio, and molecular weight of these polymers from NMR results. Although many different reactions and purification methods were attempted, none were able to fully remove PEG and achieve low polydispersity ($Đ$ < 1.2). Nevertheless, we have demonstrated that free PEG does not affect the properties studied, and that the impact of increasing PEG density could still be realized by comparing the 12% vs. 3/6% PEG-g-PGA TCN samples. 2) Large size of TCN formulations, as the size TCN (PGA) and TCN (PEG-g-PGA) was way larger than 100 nm even in Bis Tris buffer at high C:N, which means that TCN we made is not suitable for LN drainage. 3) Use pDNA instead of saRNA, as our final aim is to deliver saRNA to LN, but we chose to evaluate pDNA not mRNA or saRNA because pDNA is more stable than mRNA and saRNA and suitable for delivery platform optimization.

4.2 Future works

Future work should: 1) Develop a new method to synthesize PEG-g-PGA with monodispersity and high purity, 2) Optimize TCN mixing methods to achieve sizes that are closer to the ideal size for LN delivery (<100 nm). Future work will focus on optimizing PEG-g-PGA TCNs by increasing pABOL MW or hydrophobicity, or by decreasing PGA's charge density or MW. 3) Characterize TCN formulations with cryo-Transmission Electron Microscopy (cryo-TEM) and Fluorescence Correlation Spectroscopy (FCS) to better understand particle structure (e.g. core-shell features), and finally 4) Apply TCN formulations to mRNA or saRNA delivery with conjugated targeting ligands to achieve APC-specific nucleic acid vaccine delivery.

5 Conclusions

The present work developed a simple method to prepare pABOL TCNs with PGA or PEG-g-PGA coating polymers to overcome salt and serum-induced polyplex aggregation. Biocompatible PGA and PEG-g-PGA carboxylic acid functional groups self-assembled with positively charged polyplexes and decreased the surface charge of nanoparticles to near-neutral or even negative. Bare PGA can bind to positively charged pABOL polyplexes without unpackaging, while coating polyplexes with PEG-g-PGA led to polyplex-pDNA dissociation. We further found that pABOL polyplexes and PGA TCNs are not very size-stable in PBS and serum containing medium, while PEG-g-PGA TCNs were resistant to aggregation in such environments. The transfection efficiency of PGA

and PEG-g-PGA TCNs was high in serum-free medium but relatively poor in serum-containing medium, albeit greater than polyplexes. We hypothesize that there is a balance of PEGylation density that can limit the interaction of nanoparticles with serum proteins but have fewer negative impacts on pDNA packaging and cellular uptake. Future work will refine these promising PEGylated coating polymers to investigate nucleic acid delivery to LNs.

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