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Ligand-decorated click polypeptide derived nanoparticles for targeted drug delivery applications

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Abstract

A ligand decorated, synthetic polypeptide block copolymer platform with environment-responsive capabilities was designed. We evaluated the potential of this system to function as a polymersome for targeted-delivery of a systemic chemotherapy to tumors. Our system employed click chemistry to provide a pH-responsive polypeptide block that drives nanoparticle assembly, and a ligand (folic acid) conjugated PEG block that targets folate-receptor over-expressing cancer cells. These nanocarriers were found to encapsulate a high loading of conventional chemotherapeutics (e.g. doxorubicin at physiological pH) and release the active therapeutic at lysosomal pH upon cellular uptake. The presence of folic acid on the nanoparticle surface facilitated their active accumulation in folate-receptor-overexpressing cancer cells (KB), compared to untargeted carriers. Folate-targeted nanoparticles loaded with doxorubicin also showed enhanced tumor accumulation in folate-receptor positive KB xenografts, resulting in the suppression of tumor growth in an *in vivo* hind flank xenograft mouse model.

Keywords

Poly (propargyl L-glutamate); Drug Delivery; Nanocarriers; Block copolymers

Background

Nanoscale drug delivery systems that can release their therapeutic payload to disease-sites in response to endogenous biological cues are an active area of translational medical research^{1–4}. These engineered systems are capable of exhibiting unprecedented benefits in the management of non-metastatic cancer and solid tumors by enhancing the treatment efficiency of frontline anticancer agents, hence offsetting the need for the development of

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Author Contributions

M.A.Q and S.W.M contributed equally to this work, and the manuscript was written through contributions of all authors. M.A.Q. performed the synthesis and physicochemical analysis. S.W.M. carried out the *in vivo* experimentation and quantification of data. LBM designed the *in vitro* and immunofluorescence experiments. K.E.S. conducted TEM and cryo-TEM. JD and NE were involved in synthesis and conducting physico-chemical experiments. P.T.H. designed the experiments and supervised the project.

Notes

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newer, more expensive drug entities⁵. Amphiphilic block copolymers are a robust chemical platform that can be modularly designed to generate self-assembled nanoparticles in the form of micelles or polymersomes. They have been widely used for encapsulating a broad spectrum of potent anticancer agents through supramolecular interactions^{6–8}. The reversibility of these interactions is particularly well suited for designing stimuli-responsive drug delivery systems. Although block copolymer-based nanocarriers have been shown to prolong the circulation half-life of small-molecule drugs and promote their accumulation in diseased tissues, the inclusion of enhanced functional attributes such as microenvironment-sensitivity, active targeting capability, and programmable destabilization within these synthetic constructs calls for further molecular engineering of the constituent blocks^{9, 10}. Examples of chemical modifications of structural polymers include: (a) conjugation of small molecule ligands to the macromolecular backbone that can complementarily engage over-expressed, disease-specific cellular receptors, (b) introduction of ionizable groups within the architecture thereby allowing modulation of the surface charge of the scaffold to optimize cellular interaction and cytosolic uptake, and (c) incorporation of chemical modalities within the polymer to promote self-assembly phase-transitions in response to environmental stimuli¹¹. While designing these systems, it is necessary to control dose-limiting toxicities attributed to the premature bolus release of the drug from the delivery vehicle. Such off-target release can begin as early as the introduction of the drug carrier to the systemic environment, and indicates a certain level of instability of the carrier scaffold and dissociation of the encapsulated active drug, in most cases well before the carrier accumulates at the site of drug action.

To optimize colloidal stability and stimuli-responsive behavior, we previously designed and characterized poly (ethylene glycol)-b-poly (γ -propargyl L-glutamate) (PEG-b-PPLG) block copolymers and investigated their potential therapeutic application for environment-responsive drug and gene delivery, and as antimicrobial agents^{12–15}. A unique feature of these novel polypeptides is their biodegradable scaffold containing pendant alkyne groups. The alpha-helical arrangement of these groups makes them readily accessible for a “click” alkyne-azide cycloaddition reaction, hence enabling the generation of a large repository of biomaterials with desired form and function. In our recent studies we have shown that PEG-b-PPLG block copolymers with pH-responsive tertiary amine side chains, show pH-dependent and reversible aggregation behavior to form polymersomes (100–150 nm diameter), that can be used as endosome-solubilizing transporters of hydrophilic drug molecules¹⁶. However, this system, which relied on the tumor-associated enhanced permeation and retention (EPR) effect, showed limited selectivity in targeting tumor mice xenografts. To optimize the targeting of this nanocarrier, we have designed an orthogonal synthetic pathway to covalently link a specific molecular target onto the PPLG scaffold.

The role and molecular understanding of folic acid (FA) as a targeting modality for small molecule drug delivery systems has been extensively studied^{17, 18, 19, 20}. Owing to the high affinity of FA ($K_d \sim 0.1$ nM)¹⁹ towards glycosylphosphatidylinositol (GPI) anchored cell-surface alpha folate receptors (FR α), as well as the over-expression of these receptors in many human tumors, including tumors of the ovary²¹, uterus²², endometrium²³, brain²⁴, kidney²⁵, head and neck²⁶, and mesothelium²⁷ with limited expression on normal cells^{28, 29}, many researchers have shown that the addition of folate groups to the exterior surfaces of

nanocarriers enhances intracellular uptake in tumor cells both *in vitro* and *in vivo*, as a function of surface density balanced against PEG steric resistance^{29, 30}. Example of using folates as targeting moieties has been illustrated for drug conjugates^{31, 32}, imaging agents^{33,34}, immunotherapies^{35, 36}, liposomal assemblies^{37, 38} and polymeric nanoparticles^{39, 40}. Attachment of FA to a drug conjugate has been reported to trigger a selective uptake pathway for the drug via folate receptor mediated endocytosis and subsequent release of the conjugate from the endosome upon receptor recycling.^{41, 42} Bae et al. elegantly showed that adding folic acid in a mixed micellar system composed of poly(ethylene glycol)-b-poly (histidine) and poly (ethylene glycol)-b-poly (L-lactic acid) block copolymer forms pH-responsive nanoparticles, and when such mixed micellar systems were immobilized with folate, the nanoparticles demonstrated selective enhancement of nanoparticle entry into doxorubicin resistant MCF-7 cell lines.^{43, 44} Herein we describe the synthesis of pH-responsive PEG-b-PPLG block copolymers through a rational and facile chemical approach in which PPLG side chains are quantitatively substituted with pH-responsive tertiary alkyl amines and the PEG block is end-functionalized with folic acid. Unlike previously reported mixed micellar systems such as those prepared by Bae et al., the PEG-b-PPLG block copolymers form stable vesicles and are pH-responsive via reversible protonation of tertiary amine side chains. These newly developed block copolymers can: (i) self-assemble into nanoparticles with surface presentation of folates, (ii) encapsulate therapeutic cargo within the nanoparticle, and (iii) release the active molecule in response to a change in pH within the endosomes of folate receptor over-expressing cancer cell-lines. We investigated these folate-targeted PEG-b-PPLG nanoparticles for their dynamic self-assembly, drug encapsulation and release behavior, as well as *in vitro* cytotoxicity and selective cellular uptake in folate receptor over-expressing KB cell lines⁴⁵. We also present a mechanistic deconvolution of the intracellular trafficking mechanism of these targeted PPLG-derived system. In vivo experiments show that the particles, when administered in KB hind flank xenografts intravenously, show prolonged systemic stability, and when loaded with doxorubicin, we observe significantly improved tumor accumulation and therapeutic efficacy of the system.

Methods

Synthesis and fabrication of folate containing block copolymer vesicle

PEG-b-PPLG block copolymers terminated with tert- (butyl oxycarbonyl, BOC) (**3**) and substituted with diethyl amine side chains were synthesized as previously described (*cf.* supporting information)^{12–16}. Folate functionalized PEG-b-PPLG block copolymers with diethylamine side chain were self-assembled with unfunctionalized block copolymer at 1:10 weight ratio by diafiltration method following the methodology as described in our previous report^{16,46}(Supporting information).

In vivo experimentation: Biodistribution

For animal studies, humane care of the laboratory animals was ensured. For biodistribution studies, 0.1mL Cy 5.5 labelled folate-presenting nanoparticles were systemically administered in BALB/c mice at a concentration of 1 mg/ml. Temporally-resolved biodistribution imaging was performed using IVIS near-infrared fluorescence imaging

(Xenogen, Caliper Instruments). Necropsy of the treated mice was performed at 24h and 48h and tumor, liver, kidney, heart, spleen and lungs were harvested. Organ-wise nanoparticles and doxorubicin distribution was determined following a reported protocol⁴⁷(supporting information).

Antitumor efficacy

Antitumor efficacy was evaluated in nude mice (3–4 weeks old; Taconic firms) induced with KB tumor xenografts on hind flanks^{16, 46}. Xenografts were established by injecting 0.1 mL of 5×10^7 KB cell cells in a 1:1 cell suspension with BD Matrigel basement membrane matrix. When the tumors were palpable, animals were randomized into 3 groups (n = 3 mice per group) (day 10) and given two doses of treatment: (1) untargeted PEG-b-PPLG nanoparticles containing a dose equivalent to 5 mg/kg of doxorubicin; (2) Folate-targeted PEG-b-PPLG nanoparticles delivering 5 mg/kg doxorubicin, and; (3) 5 mg/kg free doxorubicin in PBS- all groups on day 0 (after 10 days of tumor establishment and growth phase) and on day 10. During the trial period, body weights and tumor sizes were measured once every 5 days. For tracking the accumulation of nanoparticles into the tumor region, 100 μ L of Cy 5.5 dye-labelled nanocarrier suspension were I.V. administered. Quantification of tumor size was done using Living Image ® Software. The total radiance of each tumor was determined by region of interest (ROI) analysis around the entire xenograft¹⁶.

RESULTS

Folate functionalization of amine substituted PEG-b-PPLG block copolymer

The synthetic route for generating folate-containing PEG-b-PPLG block copolymer is illustrated in Figure 1. A mono tert-BOC-protected, bis (amine) functionalized poly (ethylene glycol) (**1**) was used as the macroinitiator to mediate ring-opening polymerization of the N-carboxyanhydride (NCA) of propargyl L-glutamate (**2**). In the following step, azide terminated diethylamine was “clicked” onto the propargyl side chains by Cu-mediated azide-alkyne cycloaddition. The number average molecular weight of (**3**) was found to be ~ 14,342 Da by gel-permeation chromatography using DMF as the eluent.

Dynamic self-assembly and pH-responsive drug release behavior

Folate bearing nanoparticles optimized for cellular targeting were prepared by mixing of folic acid-functionalized (**4**), and unfunctionalized (**3**) block copolymers in 1:10 weight ratio, resulting in self-assembled nanoparticles. UV-Vis spectroscopic measurement showed that the nanoparticles contain folic acid on their surface (Figure 2A, absorption maximum at 363 nm), and based on calibration curve, approximately 7.6 ± 0.2 wt% of the nanoparticles were found to be composed of folate-bearing block copolymers. The loss of folate content (by 2.6%) in the mixed polymersome system cannot be specifically accounted for, but was most likely due to the filtration step, employed post-fabrication for improving the polydispersity of the particles (supporting information), which may selectively exclude more hydrophobic particles with higher folate content. Folate nanoparticles had a hydrodynamic diameter of 147 ± 5 nm (mean $\pm$ SD, n = 5 experimental replicate, Figure 2B) with a polydispersity index of 0.133 and a negative surface charge of -16 ± 0.3 mV (mean $\pm$ SD, n = 10). Figure 2C contains a representative transmission electron microscopy (TEM) image,

which shows spherical particles. We further confirmed that folate conjugated nanoparticles possess a vesicular structure through static light scattering (SLS), with the ratio (ρ) between radius of gyration (R_G) and radius of hydration (R_H) being 1.04. We measured a critical aggregation concentration of folate presenting polypeptide vesicles of 1.2×10^{-8} at pH 7.4 and generally observed that folate conjugation does not alter the pH-sensitive assembly and disassembly of the amine-substituted PEG-b-PPLG block copolymers (Supporting information Figure 1). At neutral pH, the folate-presenting nanoparticles were very stable, and no significant changes in hydrodynamic diameter or zeta potential were observed at 4°C for up to 3 months, analogous to poly (lactide-co-glycolide) based polymeric nanocapsules⁴⁸.

The folate-decorated nanovesicles were found to encapsulate doxorubicin with drug loading efficiency up to 25.6 wt% with final drug loading content of 14.1 wt % as measured by UV-Vis spectrophotometric methods. The cumulative release profile of loaded doxorubicin from these nanovesicles showed a pH-dependent release between pH 7.4 (PBS) and pH 5.5 buffer (Figure 2D). Doxorubicin was entrapped within the hydrophilic interior of the nanoparticles, which in serum condition (pH 7.4, 37 °C) released $15 \pm 2\%$ and $25.8 \pm 5\%$ of the initially loaded drug after 24 and 48h respectively (mean $\pm$ SD, $n = 3$). At a lower pH value of 5.5, to mimic the pH condition observed in lysosomal compartments (between pH 5.0 – 5.5), doxorubicin release from the particles was greatly accelerated (24 hours, $76 \pm 4\%$; 48 hours, $82 \pm 10\%$, mean $\pm$ SD, $n = 3$). The observed enhancement of drug release at pH 5.5 can be attributed to the protonation of the tertiary amines along the PPLG block at lower pH leading to particle destabilization as consistent with previous reports. We have compared the release of profile of targeted, doxorubicin-loaded nanoparticles with that of the non-targeted particles at pH 7.4 and 5.5. However, no statistically significant difference in $t_{25\%}$ (time required for 25% of encapsulated drug to release, for pH 7.4 and 5.5) and $t_{50\%}$ (time required for 50% release, only for pH 7.4) was observed between the two-pH conditions. As such, the pH-sensitive drug release behavior observed with the folate presenting polypeptide nanoparticles can be harnessed for targeted cancer therapy while mitigating off-target systemic toxicity related to premature release of the active drug.

Cytotoxicity and targeted cellular uptake

KB cell line has been reported in the literature as a model of folate-expressing cells therefore was selected for testing the cytotoxicity, cellular trafficking of targeted nanoparticles *in vitro*, and systemic therapeutic efficiency *in vivo*³⁰. A CCK 8 (Cell-Counting Kit-8, Dojindo Molecular Technologies, Inc.) assay was used to assess *in vitro* cytotoxicity. The cells were treated with increasing concentrations of vesicle-loaded doxorubicin for 3 days at 37 °C, and cell viability was measured at the end of the treatment to generate IC_{50} values for each of the tested formulations. Although, as observed from Figure 3A, the free drug is slightly more toxic than the nanoformulations, this trend is quite common across literature⁴⁹, and demonstrates the fact that, encapsulation within PEG-b-PPLG nanoparticles does not affect the drug's inherent toxicity in KB cell lines. At the equivalent concentration range, the cytotoxicity of block copolymers alone was also assessed in KB and in HepG2 liver cells (Figure 3B). It was found to be nontoxic to both cell lines.

In order to reveal the mechanism of binding and uptake of folate-bearing nanoparticles compared to their unfunctionalized analogue, we undertook flow-cytometric and confocal microscopy investigations⁴⁸. In this set of experiments, KB cells were grown to 70% confluence and incubated with folate-functionalized and unfunctionalized nanoparticles labeled with AF 488 for 30 minutes, 1h, or 2h at 4° C, or at 37° C, either in the absence, or presence, of excess folic acid (200 µM). Flow cytometry analysis confirmed that at 4°C – where receptor binding, but minimal endocytosis, is likely to operate – targeted nanoparticles showed enhanced cell-associated fluorescence relative to untargeted nanocarriers. Furthermore, free folic acid suppressed the uptake of the folate-bearing particles at 4 °C, providing additional evidence for the role of folate in the enhanced binding of these particles (Figure 3C). Figure 3C also illustrates that, at 37 °C, both type of particles showed enhancement in uptake for the folate-bearing nanoparticles as indicated by a substantial increase in cell-associated mean fluorescence.

In an attempt to deconvolute the mechanism of such active cellular targeting, we have conducted immunofluorescence-based experiments. Nanoparticles can be internalized by cells via receptor-mediated and non-receptor-mediated endocytosis to facilitate their intracellular delivery and augment their cellular function⁵⁰. We investigated the mechanism of intracellular trafficking of folic acid-conjugated nanoparticles in KB cells that express high levels of folate receptor alpha (FR α). To first investigate the extracellular interaction of nanoparticles, KB cells were incubated with nanoparticle suspension at different time intervals up to 30 min. The cells were stained with anti-FR α , CD44, caveolin-1, and clathrin-heavy chain antibodies to determine the pathway of cellular internalization. We observed strong cellular interactions between nanoparticles and FR α at 10min post incubation (Figure 4, Panel A, magnified in B).

Almost no significant interaction was observed for other membrane-bound receptors, confirming the successful engagement of FR α with folate-bearing PEG-b-PPLG nanoparticles and selectivity of the folic acid ligand towards the folate receptor. This data indicates folate receptor mediated endocytosis as the key mechanism for internalization of folate-bearing nanoparticles into KB cells⁵¹. To further investigate the intracellular trafficking of nanoparticles via different endosomal compartments, and whether low lysosomal pH (5.0 – 5.5) can indeed activate the pH-responsive effect of PEG-b-PPLG systems, the nanoparticles were incubated with KB cells from 30 min time intervals up to 2 h at 37° C. The cells were stained with early endosome antigen 1 (EEA-1), early to late endosomal marker (Rab7), and lysosome-associated membrane protein 1 (Lamp1). We observed the highest accumulation of nanoparticles in the early endosome, as observed by co-localization with EEA-1, at 30 min post-incubation (Figure 5, panel A, magnified in B) and at 60 min in the late endosomes as indicated by co-localization with Rab7 (Figure 5 Panel C, magnified in panel D). Reduced accumulation of nanoparticles within the lysosomal compartments, indicated by co-localization with Lamp1 at 90 min, was observed (Figure 5 Panel E, magnified in F)). We also observed a small amount of diffuse fluorescence associated with the cell cytoplasm, without localization in compartments, suggesting some degree of escape at these early stages.

We observed the intracellular pH-dependent destabilization of our nanoparticles at 120min when numerous punctate distributions of dye conjugated nanoparticles appeared around lysosomal vesicles with drastically reduced accumulation within the vesicles (Figure 6A, Panel A, magnified in B). We reasoned that the proton sponge or the pH-buffering effect of protonatable groups on the PEG-b-PPLG construct might have been activated, resulting in nanoparticles disassembling within the endosomal to lysosomal compartments (pH 5.0 – 5.5) by 120min, undergoing release from the compartment, and the subsequent re-assembly of the block copolymers into discreet nanostructures as they reach the cytosol pH of 7.4. The result of sufficient amounts of endosomal escape leads to the random aggregation of the block copolymer particles outside the lysosomal vesicles (Figure 6A, Panel A, right most image and its corresponding magnification in panel B). The observed reduced accumulation and release of nanoparticles from the lysosomal vesicles is due to endosomal escape⁵², a mechanism which allows entrapped endocytic carriers within the lysosomes to be released into the cytoplasm⁵³. In this case, the endosomes may have undergone buffering and osmotically induced release, or endosomal membrane leakage, due to interactions of the charged amines of the free polymer segments with the membrane. To confirm that the reduced accumulation and the appearance of the punctate structures around the lysosome are triggered by protonation of the nanoparticles within the organelle, we treated KB cells with low concentrations of bafilomycin A1 to specifically elevate endosomal pH to about 7.4 and incubated the cells with nanoparticles at 30min time intervals up to 2 hrs. Interestingly, we observed a reversal of the previously observed disintegration of nanoparticles from the lysosomal compartments, with increased accumulation and strong co-localization to Lamp1 signals (Figure 6, Panel C, magnified in panel D). This observation confirms that the PEG-b-PPLG nanoparticle disassembly was indeed due to the low endosomal pH and also confirms the inherent pH-responsive properties of the nanoparticles. In the absence of bafilomycin A1, at the 120min time point we observed significantly reduced quantities of nanoparticles in the lysosomal compartments in non-treated cells, suggesting gradual disintegration and escape of the nanoparticles as lysosomal pH was approached, and recycling of folate receptors from the endosomal vesicles to the extracellular membrane. These microscopy-based experiments clearly demonstrated that folic acid conjugated nanoparticles are intracellularly trafficked via folate receptor mediated endocytosis as illustrated in Figure 5b and the nanocarrier construct exhibited a pH-responsive destabilization, specifically in the lysosomes, rendering it a suitable system for delivery of small molecule drugs and inhibitors with a programmable release feature through pH induction.

The confocal microscopic images were quantified using automated Cell-Profiler pipeline to investigate the extent of colocalization with time. Box-plots (A–F) were constructed by measuring the correlation between the intensity of fluorescence signals generated from receptors as well as that generated from nanoparticles over time (Supporting information Figure 2). The y-axis ranges from –1 to +1, where –1 represents no co-localization of nanoparticles with cellular structure and +1 represents strong co-localization (n = 3 images). Fluorescence signals generated from the anti-CD44 (plot A) did not show any correlation with the nanoparticles, indicating unlikely interaction of the nanoparticles with CD44 receptors. Conversely, positive correlation between anti-FR α and nanoparticles was observed, which increased over time, then plateaued at later time points (plot B). A similar

positive association is observed between anti-EEA1 (plot **C**) and anti-Rab7 (plot **D**) with nanoparticles. Staining of cells with the lysosomal marker, antibody Lamp1 with Bafilomycin A1 treatment and Lamp1 alone are illustrated in plot **E** and **F** respectively, which show the highest positive association at later time points of 90 and 120min and plateaus thereafter. Although, Bafilomycin A1, which selectively inhibits lysosomal pH, did not affect nanoparticles co-localization completely in the lysosome within these time points, however the data points were preferentially spread towards negatively correlated area.

Biodistribution of folate-bearing presenting nanoparticles

Biodistribution of folate-bearing pH-responsive PEG-b-PPLG nanoparticles as a function of time, as well as their ability to target folate receptor-positive KB xenograft tumors, were evaluated in healthy BALB/c mice, and in nude NCR mice bearing KB tumor xenograft respectively ($n = 3$ mice per group, single flank tumor $\sim 200 \text{ mm}^3$) and then compared with an untargeted control. In healthy BALB/c mice, folate bearing, Cy 5.5 labelled nanoparticles were found to follow a two-phase pharmacokinetic decay ($\lambda_{\text{fast}} = 0.19\text{h}$, $\lambda_{\text{slow}} = 10.7\text{h}$) following systemic administration (Figure 7A). Similar observation was reported for paclitaxel-loaded liposomal structures containing folic acid as a targeting modality that showed considerably longer terminal half-lives of 12.33 h compared to the free drug (1.78 h)⁵⁴. For untargeted systems, polymeric nanoparticles showed a wide variety of circulation half-lives depending on the block composition. For example, micelles composed of poly (ethylene glycol)-b-poly (propargyl L-glutamate) with pendant hydrophobic aromatic moieties conjugated to the hydrophobic block showed a half-life of 0.12 h (fast) and 13.0 h (slow) by FRET-based measurements in a two-compartment model for blood clearance⁵⁵. On the other hand, poly (γ -benzyl L-glutamate)-block-hyaluronan polymersomes exhibited a significantly extended the half-life of 19.90 h of docataxel in circulation, as compared with the free drug, indicating the longer circulation time of block copolymer vesicles⁵⁶. A biodistribution snapshot at 24 h following systemic introduction showed $22.3 \pm 0.3\%$ ID/g (injected dose/g of tissue) in the liver, and $9.43 \pm 0.4\%$ ID/g in the kidneys, with minimal fractions detected in other organs (Figure 7B) of healthy, non-tumor bearing BALB/c mice. The observation is consistent with previous reports from Kataoka et al., where folate-conjugated, poly (ethylene glycol)-b-poly (aspartate-hydrazone-adriamycin) self-assembled systems showed strong liver accumulation⁵⁷.

It was observed in tumor bearing NCR nude mice (Figure 7C and D) that after 24h, $3.27 \pm 0.3\%$ ID/g of the targeted particles (quantified as doxorubicin dose) accumulated in the tumor, which was 1.27 fold higher ($2.56 \pm 0.3\%$ ID/g) than that exhibited by non-targeted particles ($p = 0.0198$). With an $n = 3$, the data showed that folate has limited effect on increasing nanoparticle accumulation in tumors. Although there is strong evidence that folate immobilization enhances tumor uptake in majority of xenograft models^{58, 59}, several groups showed that targeting ligand on nanoparticles does not necessarily increase nanoparticle accumulation within the tumorous tissue, but instead enhances their cellular internalization contributing to superior tumor suppression⁶⁰. In our case, the observed accumulation of non-targeted particles in tumors can be attributed to the enhanced permeation and retention (EPR) that increases the rate of particle accumulation in the tumor interstitials via “leaky vasculature”, whereas the folate-bearing vesicles are likely to

accumulate in the tumor tissue through enhanced internalization of the nanoparticles into KB cells of the xenograft. Both functionalized and unfunctionalized nanoparticles exhibited similar levels of liver accumulation after 24 h (~20–25 %ID/g, Data not shown), which was surprising, as a higher load of folate bearing nanoparticles within liver interstitials was expected. Aside from particle retention in the tumors, particle levels in all other organs sampled gradually decreased over the period of 48 h after injection, suggesting no undesirable bioaccumulation and nanomaterial excretion. The observed higher %ID/g of the nanoparticles in the liver is consistent with most systemically-administrable materials, and is likely due to enhanced phagocytosis of the particles by reticulo-endothelial systems (RES) following destabilization of the PEG-b-PPLG polymer by Kupfer cells. In addition, the presence of folate receptors in the liver, and tertiary amine side-chains present in the nanoparticle constituting polymers, might be the contributing factors responsible for the higher quantity of folate-bearing particles observed in the liver. These observations are in accordance with the behavior of folate-bearing polypeptide based nanoparticles^{30, 46} where longer and stronger retention of optimally targeted micelles in tumors 48 hours after their administration was observed.

In vivo antitumor efficacy with folate-bearing PEG-b-PPLG nanoparticles

We evaluated the efficacy of doxorubicin-loaded, folate-bearing PEG-b-PPLG nanoparticles against a KB xenograft (subcutaneous injection on hind flanks, day 0) model in NCR nude mice. When the induced tumors were ~150–200 mm³ in volume, mice were randomly divided into three groups for treatment (n = 3 mice per group). Mice receiving targeted and untargeted nanoparticles loaded with doxorubicin showed varying degrees of efficacy in slowing the growth of the xenograft compared to the control group (mice receiving free doxorubicin). After two doses of therapy, and as shown in Figure 8A, significantly smaller tumors on targeted treatment groups were observed in comparison to untargeted and free drug treated control groups (p < 0.001). Free drug delivered at the same dose (5 mg/kg) had a minimal effect in slowing tumor growth. The increased therapeutic efficiency afforded by the targeted treatment is most likely attributed to the stronger retention of targeted particles in tumors. Although the mechanism of drug release within the cancer cells from both targeted and non-targeted nanoparticles is mediated by pH-dependent protonation and subsequent particle destabilization, it is most likely that the tumor cells actively internalize folate-bearing particles in a more efficient manner compared to their non-targeted counterpart. The body weights of the mice were monitored throughout the experiment as a gross toxicity measure of the adverse dose-limiting effects of the nanoparticles (Figure 8B). No acute toxicity requiring euthanasia (body weight change > 15%) was observed for the dosages used in this study. Although all mice receiving free doxorubicin injections lost between 8% and 10% of their original weight (Figure 8B), neither group of mice injected with targeted nor non-targeted nanoparticles encapsulating doxorubicin showed significant signs of toxicity.

Discussion

In this paper we describe a facile procedure, which utilizes click-functionalizable, molecularly targeted polypeptide block copolymers that self-assemble into pH responsive

polymersomes capable of disassembly at a desired pH for controlled drug release inside target cells. These PEG based polypeptide copolymers undergo a pH-responsive protonation of the tertiary amine side chains thus showing pH-responsive assembly and disassembly behavior, and are able to encapsulate and deliver anticancer drugs to ectopically developed tumors mediated by the enhanced permeation and retention (EPR) effect¹⁶. In an attempt to impart tumor cell-specific targeting capability, we introduced targeting ligands onto the nanoparticle surface via the conjugation of the folate group to the PEG chain of a fraction of the block copolymers used for vesicular assembly. We demonstrated the ability of the nanoparticles to target cancer cells *in vitro* and *in vivo*, and to execute programmed release of drugs inside cellular microenvironment. As a model molecular ligand, we selected folic acid, which is highly relevant to ovarian cancer, and many other cancer types. Ligand-targeted nanoscale drug delivery platforms with pH-sensitive self-assembly behavior capability have proved to be a powerful strategy to achieve programmed drug accumulation in diseased tissues. In relevance to cancer, targeted delivery approaches are of critical importance, since severe non-specific toxicity of anticancer agents can significantly narrow the therapeutic window and patient compliance of existing disease management protocols. The folate-bearing nanoparticles, prepared within the scope of this study, circulated in the bloodstream with a colloidal stability that allowed them to accumulate in solid tumors. This resulted in the delivery of doxorubicin to tumors with minimal systemic toxicity, yet a significant reduction in tumor growth compared to free drug and the untargeted control. Currently we are working on the further development of targeted PEG-b-PPLG platforms for reduced opsonization and liver clearance for improved biodistribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix. Supplementary information

Supplementary data associated with this article can be found, in the online version, at doi:

Abbreviations

PEG poly (ethylene glycol)

PPLG	poly (γ -propargyl L-glutamate)
NCA	N-carboxyanhydride
CAC	Critical Aggregation Concentration
FR α	Folate receptor α
EPR	Enhanced Permeation Retention Effect

References

1. Torchillin VP. Multifunctional, stimuli-sensitive nanoparticulate systems for drug delivery. *Nature Reviews Drug Discovery*. 2014; 13:813–27. [PubMed: 25287120]
2. Petros RA, DeSimone JM. Strategies in the design of nanoparticles for therapeutic application. *Nature Reviews Drug Discovery*. 2010; 9:615–25. [PubMed: 20616808]
3. Moghimi SM, Hunter AC, Murray JC. Long-circulating and target-specific nanoparticles: theory to practice. *Pharmacology Reviews*. 2001; 53:283–318.
4. Smith DM, Simon JK, Baker JR jr. Applications of nanotechnology for immunology. *Nature Reviews Immunology*. 2013; 13:592–605.
5. Wang AZ, Langer R, Farokhzad O. Nanoparticle Delivery of Cancer Drugs. *Annual Reviews Medicine*. 2012; 63:185–98.
6. Cheng J, Teply BA, Sherifi I, Sung J, Luther G, Gu FX, Levy-Nissenbaum E, Radovic-Moreno AF, Langer R, Farokhzad OC. Formulation of functionalized PLGA-PEG nanoparticles for in vivo targeted drug delivery. *Biomaterials*. 2007; 28:869–76. [PubMed: 17055572]
7. Ahmed F, Pakunlu R, Brannan A, Bates F, Minko T, Discher DE. Biodegradable polymersomes loaded with both paclitaxel and doxorubicin permeate and shrink tumors, inducing apoptosis in proportion to accumulated drug. *Journal of Control Release*. 2006; 116:150–8.
8. Miyata K, Kakizawa Y, Nishiyama N, Harada A, Yamasaki Y, Koyama H, Kataoka K. Block cationic polyplexes with regulated densities of charge and disulfide cross-linking directed to enhance gene expression. *Journal of the American Chemical Society*. 2004; 126:2355–61. [PubMed: 14982439]
9. Zhang Q, Ko NR, Oh JK. Recent advances in stimuli-responsive degradable block copolymer micelles: synthesis and controlled drug delivery applications. *Chemical Communications*. 2012; 48:7542–52. [PubMed: 22737687]
10. Oltra NS, Nair P, Discher DE. From stealthy polymersomes and filomicelles to “self” Peptide-nanoparticles for cancer therapy. *Annual Review Chemical and Biomolecular Engineering*. 2014; 5:281–99.
11. Chauhan VP, Jain RK. Strategies for Advancing Cancer Nanomedicine. *Nature Materials*. 2013; 12:958–62. [PubMed: 24150413]
12. Engler AC, Lee HI, Hammond PT. Highly efficient “grafting onto” a polypeptide backbone using click chemistry. *Angew Chem Int Ed Engl*. 2009; 48:9334–8. [PubMed: 19902445]
13. Engler AC, Shukla A, Puranam S, Buss HG, Jreige N, Hammond PT. Effects of side group functionality and molecular weight on the activity of synthetic antimicrobial polypeptides. *Biomacromolecules*. 2011; 12:1666–74. [PubMed: 21443181]
14. Zhao X, Poon Z, Engler AC, Bonner DK, Hammond PT. Enhanced stability of polymeric micelles based on postfunctionalized poly(ethylene glycol)-b-poly(γ -propargyl L-glutamate): the substituent effect. *Biomacromolecules*. 2012; 13:1315–22. [PubMed: 22376183]
15. Engler AC, Bonner DK, Buss HG, Cheung EY, Hammond PT. The synthetic tuning of clickable pH responsive cationic polypeptides and block copolypeptides. *Soft Matter*. 2011; 7:5627–37.
16. Quadir MA, Morton SW, Deng ZJ, Shopsowitz KE, Murphy RP, Epps TH, Hammond PT. PEG-polypeptide block copolymers as pH-responsive endosome-solubilizing drug nanocarriers. *Molecular Pharmaceutics*. 2014; 11:2420–30. [PubMed: 24813025]

17. Xia W, Low PS. Folate-targeted therapies for cancer. *J Med Chem.* 2010; 53:6811–24. [PubMed: 20666486]
18. Leamon CP. Folate-targeted chemotherapy. *Adv Drug Delivery Rev.* 2004; 56:1127–41.
19. Hilgenbrink AR, Low PS. Folate receptor-mediated drug targeting: From therapeutics to diagnostics. *J Pharm Sci.* 2005; 94:2135–46. [PubMed: 16136558]
20. Chen C, Jiyuan K, Zhou XE, Wei Y, Joseph SB, Jun L, Eu-Leong Y, Xu HE, Karsten M. Structural basis for molecular recognition of folic acid by folate receptors. *Nature Materials.* 2013; 500:486–89.
21. O'Shannessy DJ, Somers EB, Wang LC, Wang H, Hsu R. Expression of folate receptors alpha and beta in normal and cancerous gynecologic tissues: correlation of expression of the beta isoform with macrophage markers. *J Ovarian Res.* 2015; 8
22. Allard JE, Risinger JI, Morrison C, Young G, Rose GS, Fowler J, Berchuck A, Maxwell GL. Overexpression of folate binding protein is associated with shortened progression-free survival in uterine adenocarcinomas. *Gynecol Oncol.* 2007; 107
23. Brown Jones M, Neuper C, Clayton A, Mariani A, Konecny G, Thomas MB, Keeney G, Hartmann L, Podratz KC. Rationale for folate receptor alpha targeted therapy in “high risk” endometrial carcinomas. *Int J Cancer.* 2008; 123:1699–703. [PubMed: 18646191]
24. Weitman SD, Frazier KM, Kamen BA. The folate receptor in central nervous system malignancies of childhood. *J Neurooncol.* 1994; 21:107–12. [PubMed: 7861186]
25. Parker N, Turk MJ, Westrick E, Lewis JD, Low PS, Leamon CP. Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. *Anal Biochem.* 2005; 338:284–93. [PubMed: 15745749]
26. Saba NF, Wang X, Müller S, Tighiouart M, Cho K, Nie S, Chen Z, Shin DM. Examining expression of folate receptor in squamous cell carcinoma of the head and neck as a target for a novel nanotherapeutic drug. *Head Neck.* 2009; 31:475–81. [PubMed: 19072997]
27. Yuan Y, Nymoen DA, Dong HP, Bjørang O, Shih LM, Low PS, Trope' CG, Davidson B. Expression of the folate receptor genes FOLR1 and FOLR3 differentiates ovarian carcinoma from breast carcinoma and malignant mesothelioma in serous effusions. *Hum Pathol.* 2009; 40:1453–60. [PubMed: 19454358]
28. Bandara NA, Hansen MJ, Low PS. Effect of receptor occupancy on folate receptor internalization. *Molecular Pharmaceutics.* 2014; 11:1007–13. [PubMed: 24446917]
29. van Dongen MA, Silpe JA, Dougherty CA, Kanduluru AK, Choi SK, Orr BG, Low PS, Banaszak Holl MM. Avidity mechanism of dendrimer-folic acid conjugates. *Molecular Pharmaceutics.* 2014; 11:1696–706. [PubMed: 24725205]
30. Poon Z, Chen S, Engler AC, Lee HI, Atas E, von Maltzahn G, Bhatia SN, Hammond PT. Ligand-clustered “patchy” nanoparticles for modulated cellular uptake and in vivo tumor targeting. *Angew Chem Int Ed Engl.* 2010; 49:7266–70. [PubMed: 20740515]
31. Wayua C, Roy J, Putt KS, Low PS. Selective Tumor Targeting of Desacetyl Vinblastine Hydrazide and Tubulysin B via Conjugation to a Cholecystokinin 2 Receptor (CCK2R) Ligand. *Mol Pharm.* 2015; 12:2477–83. [PubMed: 26043355]
32. Zong H, Thomas TP, Lee K-H, Desai AM, Li M-h, Kotlyar A, Zhang Y, Leroueil PR, Gam JJ, Banaszak Holl MM, Baker JR. Bifunctional PAMAM Dendrimer Conjugates of Folic Acid and Methotrexate with Defined Ratio. *Biomacromolecules.* 2012; 13:982–91. [PubMed: 22360561]
33. De Jesus E, Keating JJ, Kularatne SA, Jiang J, Judy R, Predina J, Nie S, Low P, Singhal S. Comparison of Folate Receptor Targeted Optical Contrast Agents for Intraoperative Molecular Imaging. *Int J Mol Imaging.* 2015; 2015:469047. [PubMed: 26491562]
34. Liong M, Lu J, Kovichich M, Xia T, Ruehm SG, Nel AE, Tamanoi F, Zink JJ. Multifunctional Inorganic Nanoparticles for Imaging, Targeting, and Drug Delivery. *ACS Nano.* 2008; 2:889–96. [PubMed: 19206485]
35. Kelderhouse LE, Robins MT, Rosenbalm KE, Hoylman EK, Mahalingam S, Low P. Prediction of Response to Therapy for Autoimmune/Inflammatory Diseases Using an Activated Macrophage-Targeted Radioimaging Agent. *Mol Pharm.* 2015; 12:3547–55. [PubMed: 26333010]
36. Lu Y, Sega E, Leamon CP, Low PS. Folate receptor-targeted immunotherapy of cancer: mechanism and therapeutic potential. *Adv Drug Delivery Rev.* 2004; 56:1161–76.

37. Poh S, Chelvam V, Low PS. Comparison of nanoparticle penetration into solid tumors and sites of inflammation: studies using targeted and nontargeted liposomes. *Nanomedicine*. 2015; 10:1439–49. [PubMed: 25996118]
38. Gabizon A, Horowitz AT, Goren D, Tzemach D, Mandelbaum-Shavit F, Qazen MM, Zalipsky S. Targeting Folate Receptor with Folate Linked to Extremities of Poly(ethylene glycol)-Grafted Liposomes: In Vitro Studies. *Bioconjugate Chem*. 1999; 10:289–98.
39. Bandara NA, Hansen MJ, Low PS. Effect of receptor occupancy on folate receptor internalization. *Mol Pharm*. 2014; 11:1007–13. [PubMed: 24446917]
40. Quintana A, Raczka E, Piehler L, Lee I, Myc A, Majoros I, Patri A, Thomas T, Mulé J, Baker J Jr. Design and Function of a Dendrimer-Based Therapeutic Nanodevice Targeted to Tumor Cells Through the Folate Receptor. *Pharm Res*. 2002; 19:1310–16. [PubMed: 12403067]
41. Leamon CP, Low PS. Delivery of macromolecules into living cells: a method that exploits folate receptor endocytosis. *Proc Natl Acad Sci USA*. 1991; 88:5572–76. [PubMed: 2062838]
42. Kamen BA, Capdevila A. Receptor-mediated folate accumulation is regulated by the cellular folate content. *Proc Natl Acad Sci USA*. 1986; 83:5983–87. [PubMed: 3461471]
43. Lee ES, Na K, Bae YH. Doxorubicin loaded pH-sensitive polymeric micelles for reversal of resistant MCF-7 tumor. *Journal of Control Release*. 2005; 103:405–18.
44. Lee ES, Na K, Bae YH. Polymer micelle for tumor pH and folate mediated targeting. *Journal of Controlled Release*. 2003; 91
45. Lu Y, Sega E, Leamon CP, Low PS. Folate receptor-targeted immunotherapy of cancer: mechanism and therapeutic potential. *Advanced Drug Delivery Reviews*. 2004; 56:1161–76. [PubMed: 15094213]
46. Poon Z, Lee JA, Huang S, Prevost RJ, Hammond PT. Highly stable, ligand-clustered “patchy” micelle nanocarriers for systemic tumor targeting *Nanomedicine: Nanotechnology, Biology and Medicine*. 2011; 7:201–09.
47. Maltzahn, Gv, Park, J-H., Lin, KY., Singh, N., Schwöppe, C., Mesters, R., Berdel, WE., Ruoslahti, E., Sailor, MJ., Bhatia, SN. Nanoparticles that Communicate In Vivo to Amplify Tumour Targeting. *Nature Materials*. 2011; 10:545–52. [PubMed: 21685903]
48. El-Gogary RI, Rubio N, Wang JT-W, Al-Jamal WT, Bourgognon M, Kafa H, Naeem M, Klippstein R, Abbate V, Leroux F, Bals S, Tendeloo GV, Kamel AO, Awad GAS, Mortada ND, Al-Jamal KT. Polyethylene Glycol conjugated polymeric nanocapsules for targeted delivery of quercetin to folate-expressing cancer cells. *ACS Nano*. 2014; 8:1384–401. [PubMed: 24397686]
49. Yang Y, Pan D, Luo K, Li L, Gu Z. Biodegradable and amphiphilic block copolymer-doxorubicin conjugate as polymeric nanoscale drug delivery vehicle for breast cancer therapy. *Biomaterials*. 2013; 34:8430–43. [PubMed: 23896006]
50. Reinecke J, Caplan S. Endocytosis and the Src family of non-receptor tyrosine kinases. *Biomol Concepts*. 2014; 5:143–55. [PubMed: 25372749]
51. Lu T, Sun J, Chen X, Zhang P, Jing X. Folate-conjugated micelles and their folate-receptor-mediated endocytosis. *Macromol Biosci*. 2009; 9:1059–68. [PubMed: 19688723]
52. Varkouhi AK, Scholte M, Storm G, Haisma HJ. Endosomal escape pathways for delivery of biologicals. *J Control Release*. 2011; 151:220–8. [PubMed: 21078351]
53. Wong AS, Mann SK, Czuba E, Sahut A, Liu H, Suekama TC, Bickerton T, Johnston AP, Such GK. Self-assembling dual component nanoparticles with endosomal escape capability. *Soft Matter*. 2015; 11:2993–3002. [PubMed: 25731820]
54. Wu J, Liu Q, Lee RJ. A folate receptor-targeted liposomal formulation for paclitaxel. *Int J Pharmaceutics*. 2006; 316:148–53.
55. Morton SW, Zhao X, Quadir MA, Hammond PT. FRET-enabled Biological Characterization of Polymeric Micelles. *Biomaterials*. 2014; 35:3489–96. [PubMed: 24477190]
56. Upadhyay KK, Bhatt AN, Castro E, Mishra AK, Chuttani KC, Dwarakanath BS, Schatz C, Le Meins J-F, Misra A, Lecommandoux S. In vitro and In vivo Evaluation of Docetaxel Loaded Biodegradable Polymersomes. *Macromol Biosci*. 2010; 10:503–12. [PubMed: 20232310]
57. Bae Y, Nishiyama N, Kataoka K. In Vivo Antitumor Activity of the Folate-Conjugated pH-Sensitive Polymeric Micelle Selectively Releasing Adriamycin in the Intracellular Acidic Compartments. *Bioconjugate Chem*. 2007; 18:1131–39.

58. Gabizon A, Horowitz AT, Goren D, Tzemach D, Shmeeda H, Zalipsky S. In vivo fate of folate-targeted polyethylene-glycol liposomes in tumor-bearing mice. *Clin Cancer Res.* 2003; 9:6551–59. [PubMed: 14695160]
59. Yamada A, Taniguchi Y, Kawano K, Honda T, Hattori Y, Maitani Y. Design of folate-linked liposomal doxorubicin to its antitumor effect in mice. *Clin Cancer Res.* 2008; 14:8161–68. [PubMed: 19088031]
60. Oyewumi MO, Yokel RA, Jay M, Coakley T, Mumper RJ. Comparison of cell uptake, biodistribution and tumor retention of folate-coated and PEG-coated gadolinium nanoparticles in tumor-bearing mice. *J Control Release.* 2004; 95:613–26. [PubMed: 15023471]

Figure 1.
Synthetic steps towards the preparation of folate immobilized PEG-b-PPLG systems.

Figure 2.

(A) UV-Visible spectrum of folic acid decorated nanoparticle suspension indicates the immobilization of folic acid as evident by the characteristic shoulder of folic acid at 363 nm (B) Folate functionalized nanoparticles have a hydrodynamic diameter of 147 nm, based on intensity and measured in PBS at 25°C (C) TEM image of the vesicles formed at pH 8.0 with a polymer concentration of 1 mg ml⁻¹, and dried on a Cu grid. The scale bar for the TEM image is 500 nm (D) Cumulative release profile of doxorubicin from nanoparticles in PBS (pH 7.4) and citrate buffer of pH 5.5.

Figure 3. Efficacy evaluation of vesicle-loaded doxorubicin in cells: (A) Concentration-dependent cell death of KB cells by the nanoparticles relative to free doxorubicin (B) Cytotoxicity of the empty vesicles where the concentrations of the vesicles were normalized to their corresponding doxorubicin loading as that of (A). The experiments were performed in triplicate. Data are presented as the mean $\pm$ standard deviation. (C) Flow-cytometry cell association profile of folate-functionalized and unfunctionalized PEG-b-PPLG nanoparticles in KB cells. Mean fluorescence intensity decreases statistically when folate-functionalized nanoparticles are incubated with KB cells in ligand excess conditions.

Figure 4.

KB cells were incubated with 0.1mg/mL of Alexa Fluor 488 labelled folate functionalized PEG-b-PPLG nanoparticles for 10 minutes. KB cells, which have a high level of folate receptor alpha expression, showed strong extracellular interaction with nanoparticles at 10 min post-incubation. Panel B is a zoomed-in version of the corresponding cells marked in Panel A.

Figure 5.

Nanoparticles accumulated in the early endosome as evidenced by strong co-localization to EEA-1 at 30min (Panel A, magnified in panel B). At 60min post-incubation nanoparticles were transported in the late endosome and showed strong co-localization with late endosome marker, Rab7 (Panel C, magnified in panel D). At 90min post-incubation, nanoparticles start appearing in lysosomes as evidenced by their co-localization with lysosomal marker (Lamp 1) (Panel E, magnified in panel F).

Figure 6.
 (A) Reduced accumulation and localization was observed in the lysosome vesicles (possibly due to pH-responsive disintegration, punctate formation and precipitation of PEG-b-PPLG outside the lysosomal environment pH 5.0 (Panel A, magnified in panel B). This process of nanoparticles disintegration was completely reversed by modulating the low pH in lysosome with Bafilomycin A1 to pH 7.4 (Panel C, magnified in panel D). Images were captured on Olympus DeltaVision fluorescent microscope with $\times 60$ and $\times 100$ oil objective. Scale bars correspond to $20\mu\text{m}$. (B) Schematic representation of the purported mechanism of entry of PEG-b-PPLG block copolymeric nanoparticles inside cytosol.

Figure 7. (A) Tertiary amine substituted, folic acid decorated nanoparticles (labelled with Cy 5.5) are stable in the blood stream for 24 h following systemic administration in non-tumor bearing BALB/c mice. (B) Biodistribution quantitation 24 h following systemic administration in BALB/c mice (C) Biodistribution of folate bearing nanoparticles in tumor bearing nude mice at 24 h post-systemic administration (left image: biodistribution of untargeted nanoparticles; right image: folic acid targeted vesicles) (D) Comparative tumor accumulation of targeted and untargeted nanoparticles in NCR nude mice.

Figure 8.

(A) Tumor remediation study ($n = 3$) against KB xenografts in NCR nude mice, comparing untreated free-doxorubicin-treated and drug-loaded targeted, and untargeted PEG-b-PPLG nanoparticles groups after two successive doses on 0 and 10 days. (B) No significant toxicity in terms of body weight change was evident in case of folate-bearing, targeted or untargeted nanoparticles in tumor-bearing mice ($n = 3$).