Development of a Pemetrexed/Folic Acid Nanoformulation: Synthesis, Characterization, and Efficacy in a Murine Colorectal Cancer Model

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ABSTRACT: The folate analogue pemetrexed (PEM) is an approved therapeutic for non-small cell lung cancer and malignant pleural mesothelioma with the potential for broader application in combination therapies. Here, we report the development of a nanoformulation of PEM and its efficacy against the CT26 murine colorectal cancer cell line in vitro and in vivo. Utilizing layer-by-layer deposition, we integrate PEM, along with folic acid (FA), onto a fluorescent polystyrene nanoparticle (NP) substrate. The final nanoformulation (PEM/FA-NP) has a size of ∼40 nm and a zeta potential of approximately −20 mV. Cell uptake studies indicated increased uptake in vitro for the PEM/FA-NP compared to the uncoated NP, likely due to the presence of PEM and FA. Viability studies were performed to determine the potency of the PEM/FA-NP formulation against CT26 cells. Syngeneic CT26 tumors in BALB/c mice showed reduced growth when treated once daily (2.1 mg/kg PEM) for 3 days with PEM/FA-NP versus the vehicle (uncoated) control, with no observable signs of systemic toxicity associated with the nanoformulation. Although the current study size is limited (n = 4 animals for each group), the overall performance and biocompatibility of the PEM/FA-NP observed suggest that further optimization and larger-scale studies may be warranted for this novel formulation.

INTRODUCTION

The delivery of chemotherapy by various nanocarriers presents a myriad of advantages from a theoretical standpoint.1 The nanocarriers themselves can be biocompatible2,3 and allow for combination with other modalities to allow for diagnostics or synergistic interactions with other common cancer therapies.4−8 Targeted drug delivery can be approached through active or passive mechanisms. In the context of cancer, passive targeting strategies generally refer to size-based vascular retention and the potential to exploit phenomena, such as the enhanced permeability and retention (EPR) effect.9 Active targeting generally requires molecular recognition with a surface receptor present on a target.1,9 Nanocarriers can be assembled in such a way to tailor the terminal surface to those specific biomarkers.10 Folic acid (FA) is an essential component in DNA replication,11,12 and requirements for it increase in highly proliferating cells, such as cancer. Targeting various folate receptors, including the alpha-folate receptor and reduced folate carrier, emerged as an important therapeutic target as a result.12 Folic acid has also been used as a targeting agent in various formulations previously, via direct attachment to a therapeutic,13,14 or through the use of a nanocarrier.9,15,16 Furthermore, folate antimetabolites were developed as a class of chemotherapeutics involved in impairing rapid proliferating cells by interfering in the folate pathway.11,17,18 Here, we investigate the nanoformulation of pemetrexed (PEM), which inhibits thymidylate synthase, dihydrofolate reductase, and glycaminide ribonucleotide formyltransferase, three key enzymes required for DNA replication.19,20 The interruption of these pathways leading to DNA replication can be mechanistically important against highly proliferating cells in cancer.4 Pemetrexed is an approved therapeutic agent for non-small cell lung cancer (NSCLC) and mesothelioma21 and has also been investigated in combination with carboplatin and pembrolizumab for the treatment of NSCLC.22 However, the drug runs into toxicity issues at higher doses.20 To address this issue, PEM has been formulated with various carriers and delivery strategies.4,23−25 The drug itself has higher affinity for the alpha-folate receptor than FA12 and similar affinity to raltitrexed and methotrexate in L1210 cells.12 In this work, we developed a novel nanoparticle (NP) formulation of PEM with FA (PEM/FA-NP) and evaluated its
efficacy as treatment for colorectal cancer (CRC) in a preclinical model. Previously, we observed the effect of a similar anti-metabolite therapeutic, raltitrexed, when formulated onto a polystyrene substrate via layer-by-layer (LbL) deposition. However, here, we build on previous studies by examining the efficacy of PEM/FA-NP versus the CT26 murine CRC model in vitro and in vivo. By using both PEM and FA as targeting agents, we aim to potentially see higher efficacy due to higher uptake in vivo. The facile and modular nature of LbL deposition has been used to develop a variety of therapeutics for drug delivery. In this report, we present a method to deposit both PEM and FA onto a polystyrene latex (CML) nanoparticles and again purified via tangential flow filtration (TFF). Second, pemetrexed (PEM) and folic acid (FA) were mixed with the PLL-coated nanoparticles and again purified with TFF, yielding the layered product.

RESULTS AND DISCUSSION

Nanoparticle Formulation and Characterization. Polyelectrolyte (PE) layers of PLL and PEM/FA were deposited onto the surface of carboxylate-modified polystyrene latex (CML) cores to form functional NPs. This process is illustrated in Figure 1.

![Figure 1](https://dx.doi.org/10.1021/acsomega.0c01550)

**Figure 1.** Diagram of the layer-by-layer process. First, poly-L-lysine (PLL) is mixed with the carboxylate-modified polystyrene latex (CML) nanoparticle substrate and purified via tangential flow filtration (TFF). Second, pemetrexed (PEM) and folic acid (FA) are mixed with the PLL-coated nanoparticles and again purified via TFF, yielding the layered product.

Simple mixing of the NPs with the oppositely charged layer allows for interaction and attachment followed by tangential flow filtration (TFF) that allows for rapid clearance of excess PE. While this can be done with centrifugation with larger particles, generally, >100 nm diameter cores are needed to be able to separate the NPs out of the mixture. Ultra-centrifugation can accomplish separation of smaller NPs but is generally slower and can require multiple washes to remove excess PE. Previous works had shown the usefulness of TFF for similar systems. Nanoparticle formulations were characterized using transmission electron microscopy (TEM) and dynamic light scattering (DLS) following purification of excess PEM and FA. TEM images of the PEM/FA-NP product are shown in Figure 2A.

The lower-magnification image displays multiple NPs, while the inset shows a higher-magnification image of a single PEM/FA-coated particle. A previous work had shown contrast differences in multilayers added to larger particles, however, with a single multilayer added to a much smaller substrate and with a small molecule constituting the terminal layer, the addition of the layers is difficult to distinguish even by electron microscopy. DLS was used to further characterize the size of the NPs. A sample population distribution (gained by intensity measurement) for the finished product is shown in Figure 2B. Intensity-averaged particle size, or Z-Ave, measurements showed particle size growth with the addition of the PE, while concurrent zeta potential measurements showed surface charge changes. These measurements are shown in Figure 2C. The PEM/FA final formulation had a Z-Ave of 42.77 ± 2.29 nm, with a zeta potential of −21.27 ± 0.49 mV. The addition of the PEM/FA layer does not appear to add any discernible size to the NP, which could be expected, as both PEM and FA are small molecules with relatively low molecular weights (in comparison to PLL, which is multiple orders of magnitude higher by mass). The weighted average size of the nanoparticles does appear to decrease but is not significantly different. With different amounts of aggregates detected at different points in measurements, slight variations like this are possible. Again, the terminal layer is composed of small molecules, meaning the terminal layer should not be imparting a significant detectable size change in the final product. The zeta potential does revert back to an overall negative charge, indicating that the PEM/FA is present on the surface. To investigate the presence of the desired molecules on the surface of the particle, infrared spectroscopy was employed. In Figure 2D, waveforms for the uncoated particle and the PEM/FA-NP are shown. A broad peak is observed at 3000−3500 cm⁻¹, perhaps indicating latent moisture in the sample or partially obscuring peaks indicating the presence of the carboxylic acids. The large and medium peaks between 1450 and 1650 cm⁻¹ are likely attributed to the amine (≈1500 to 1600 cm⁻¹) and amide (≈1610−1650 cm⁻¹) functional groups that are not present in the uncoated polystyrene core, of which the contributions from the individual molecules can be observed in Figure S1. To further confirm the presence of PEM and FA on the particle and to estimate their concentrations in the formulation, HPLC was used. Initial analysis indicated that by collecting absorbance and fluorescence data from the samples, the concentration of FA could be determined independently of PEM via fluorescence. This was done by correlation to an absorbance value, which could be used to separate the overlapping signal of FA from the PEM/FA absorbance mixtures. This was necessary as the retention times of PEM and FA under our experimental conditions were near identical, leading to an overlapping in the peak areas for the small molecules. PEM alone did not show fluorescence under the conditions used, allowing for separation of the absorbance signal from the PEM/FA mixture’s overlapped signal. This method is similar to a method used in the literature used to discern folate from other similar molecules. To determine the concentration of PEM and FA present in the final formulation, the PLL-coated particles were mixed with the PEM/FA mixture normally, and this mixture was transferred to a 100 kDa spin filtration unit. Instead of purifying via TFF, the NPs were separated from excess PEM/FA using this spin filtration unit. The filtrate was then loaded into a 96-well plate for use with the HPLC instrument. Samples from the filtrate indicated the amount of unloaded PEM and FA, which could be subtracted from the initial amount of PEM and FA added to the mixture, indicating the
amount of PEM and FA that had been loaded into the NP formulation. From HPLC, the concentration of PEM present in the PEM/FA-NP formulation was determined to be 213.5 μg/mL, indicating an encapsulation efficiency of 51.3%. The concentration of FA was 68.0 μg/mL, which gave an encapsulation efficiency of 16.3%. This difference could be attributed to a few different effects, including issues in mixing the two solutions (of FA and PEM), and different affinities of the two molecules for attachment to the surface of the PLL-NP. To evaluate the robustness of the formulation process, a similarly designed NP with only PEM present in the terminal layer was formulated for comparison and was found to contain a PEM concentration of 185.6 μg/mL (an encapsulation efficiency of 73.1%). Additional details about the characterization of this PEM-NP formulation are presented in Figure S1.

Cell Uptake and Viability. The cell uptake and internalization of the PEM/FA-NP formulation versus the bare particle, or vehicle control, were evaluated by flow cytometry and fluorescence microscopy. After 4 h incubation with CT26 CRC cells, the NP formulations were aspirated, washed, and prepared for evaluation. In the case of flow cytometry, the cells were detached from the wells using trypsin, dispersed and pelleted in media, fixed and pelleted in 10% formalin/PBS, and washed with/dispersed in PBS for flow cytometry. Cell populations were assessed for fluorescence intensity using a 632 nm excitation laser with a 655−730 nm emission filter for the 660/680 dye present in the various formulations using a MACSQUANT flow cytometer (Miltenyi Biotec). The mean fluorescence intensities of the wells are found in Figure 3A.
with the population data from representative wells for each NP and control shown in Figure 3B.

The no treatment control, which received no NPs, shows a low background signal in the cells. The bare NPs and PEM/FA-NPs show populations with fluorescence intensity on the same order, with the PEM/FA-NP average signal around two to three times the intensity of the bare particles. The bare particles are more narrowly distributed, with the PEM/FA-NPs showing a slightly wider intensity range of the population. This same trend was observed in a similar system of raltitrexed/folic acid NPs in a previous work, although raltitrexed/folic acid NPs showed higher uptake in the same cell line under the same conditions.

For a qualitative visualization of the uptake via fluorescence microscopy, the cells were fixed in the 6-well plates using 10% formalin/PBS, washed with PBS, and stained with DAPI and AlexaFluor 488-phalloidin. The fluorescence images were taken on an EVOS FL Auto II (ThermoFisher). The images taken for each of the treatment conditions are shown in Figure 4.

Qualitatively, the brightness of the red signal correlates with the treatment used in the same manner observed with flow cytometry. Also, similar to the flow cytometry results, the same trend was observed in a previous work with microscopy. The PEM-NP formulation, for comparison, was showed similar fluorescence values (Figure S2).

To assess toxicity versus the CT26 cells in vitro, an Alamar Blue cell viability assay was used. Cells were plated in 96-well plates, dosed, and, after 72 h, washed. The reagent, Alamar Blue, was added with fresh media and allowed to incubate for 90 min. After this period, the plates were read in a plate reader. After accounting for the background signal, the individual wells were compared to the average of the untreated control wells. This gave a percentage of viable cells, which increases with decreasing amount of therapeutic. In this assay, the dose response of the uncoated NPs is compared to the dose response of the PEM/FA-NPs (Figure 5A).

The uncoated particles show little to no toxicity across the concentration range, while the PEM/FA-NPs are efficacious, with an IC_{50} value of 0.031 μg/mL (in terms of the concentration of CML). The assay was also run to compare with the free drug, with the results shown in Figure 5B. The NPs showed an IC_{50} value of 0.33 μg/mL (concentration of PEM). For comparison, the free drug IC_{50} was found to be 0.013 μg/mL against the same CT26 cell line (free drug indicates a solution containing non-encapsulated PEM). A similar trend was seen with raltitrexed in our previous formulation, where the free drug IC_{50} was an order of magnitude less than the NP formulation. Additionally, the PEM-NP formulation showed similar viability trends and IC_{50} values and is shown in Figure S2. The [PEM] viability curve for the PEM-NPs falls closely in line with the PEM/FA NPs, with a slightly lower IC_{50} value of 0.21 μg/mL, indicating slightly higher potency of this formulation. The
difference between the NP curves and the free PEM curve can be attributed to this being an in vitro assay, where the free drug has complete access to the target, but also uptake differences between the small molecule and the NP and release of the drug from the NP once exposed to the cells. This could also be due to our method for quantifying the amount of drug loading as we indirectly account for the amount of PEM on the NPs through determination of unloaded PEM in the filtrate. A more direct method of measurement would likely indicate that the loading is less than what is reported here and would reduce the gap between the two curves. While the uptake and viability of the PEM/FA NP appear to indicate that the formulation containing folic acid may be slightly less effective than other formulations, an earlier study had indicated that the presence of folic acid may lead to enhanced efficacy in vivo.

**In Vivo Tumor Efficacy.** Efficacy of the PEM/FA-NP formulation was evaluated versus the vehicle (bare NP) control. BALB/c mice (6–8 weeks old) were injected with CT26 cells in the hind flank and allowed to grow for 2 weeks. Once tumors were of measurable size (three times weekly for the observation period), animals were divided into the three groups and injected once daily for 3 days with either bare NPs or PEM/FA-NPs. The tumor growth curves for each animal arranged by group are shown in Figure 6A,B.

Of interest here is the PEM/FA-NP group, in which the application of the nanoformulation appears to exhibit an inhibitory effect on the tumor growth, which was indicated by the in vitro viability testing. When comparing the PEM/FA formulation to the bare carrier, nearly all animals showed a reduced overall growth trend, with one animal not responding to the treatment and showing rapid tumor growth. This could be due to the small molecule and the NP and release of the drug from the NP once exposed to the cells. This could also be due to our method for quantifying the amount of drug loading as we indirectly account for the amount of PEM on the NPs through determination of unloaded PEM in the filtrate. A more direct method of measurement would likely indicate that the loading is less than what is reported here and would reduce the gap between the two curves. While the uptake and viability of the PEM/FA NP appear to indicate that the formulation containing folic acid may be slightly less effective than other formulations, an earlier study had indicated that the presence of folic acid may lead to enhanced efficacy in vivo.

Figure 6. Small-scale efficacy study of the nanoformulation. Individual tumor growth curves for (A) bare NP-treated and (B) PEM/FA-NP-treated groups (n = 4). (C) Average tumor volumes 8 days (top) and 10 days (bottom) after treatment show marked inhibition of tumor growth for three of four animals in the PEM/FA-NP group. Bars indicate mean ± standard deviation. Data significance was evaluated with unpaired Student’s t-test, ns = not significant, *P < 0.05. (D) Median survival curves for the PEM/FA-NP versus the bare NP groups.
To assess systemic toxicity associated with the PEM/FA treatment, animal weights were tracked, and organs slices were examined for signs of toxicity (Figure 7).

Animals in the PEM/FA-NP group did not show a significant reduction in average weight, indicating tolerance of the drug-loaded particles during the treatment period, as shown in Figure 7A and Figure S5. No discernible differences in histology were observed in the control groups versus the treatment group, which in this case indicates that the drug treatment is likely not causing any short-term toxicity in the clearance organs (Figure 7B).

■ CONCLUSIONS

In this work, the efficacy of a dual-loaded PEM/FA-NP against the CT26 murine CRC cell line is evaluated. The nanoformulation was constructed using a 20 nm fluorescent polystyrene substrate, onto which a layer of PLL was deposited, followed by addition of PEM and FA. The formulation showed enhanced uptake in the CT26 cells in vitro and toxicity against this cell line through a viability assay. The PEM/FA-NP formulation seemed to show a moderate increase in tumor growth inhibition when administered three times over 3 days to animals with CT26 tumors without short-term systemic toxicity. Future experiments that involve larger times over 3 days to animals with CT26 tumors without short-term systemic toxicity will be needed to determine the in vivo activity of the PEM/FA-NP formulation.

■ EXPERIMENTAL SECTION

Materials. Pemetrexed disodium heptahydrate and poly-L-lysine hydrochloride (PLL; 15−30 kDa) were purchased from Sigma Aldrich. Folic acid was purchased from Fisher Scientific. All LbL methods were carried out in Milli-Q water from a Milli-Q source. The KR2i TFF system was purchased from Spectrum Laboratories (Repligen, Waltham, MA). The K20i TFF system was purchased from Krueger Laboratories (Shimadzu, Streamline, CA). Drawn aliquots were run dropwise. Following dropwise addition of NP solution to the grid, the grids were placed in a desiccator overnight to allow the surface to dry. Infrared spectroscopy was carried out using a Nicolet iS5 spectrophotometer (ThermoFisher, Waltham, MA). Freshly prepared NP solutions were lyophilized, and the dry powder was compressed into a diamond filament. Total internal reflectance measurements were used to acquire the spectrum for each formulation at 32X resolution between 4000 and 400 cm−1.

Drug loading and encapsulation efficiency were determined via high-performance liquid chromatography (HPLC). To determine the drug content, solutions containing PEM/FA-NP with excess polyelectrolytes were passed through a 10 kDa spin filtration unit (Pall, New York, NY). The samples were spun at 5500 rpm for 5 min, which allowed the drug to exit as a filtrate while retaining the NPs in the retentate. Filtrate samples were run on a Shimadzu SPD-20A HPLC instrument (Shimadzu, Torrance, CA) equipped with a UV−Vis detector and an Agilent Zorbax Rapid Resolution SBC-18 column (4.6 × 100 mm, 3.5 μm, Santa Clara, CA). Drawn aliquots were run without dilution. A 5 min gradient method was utilized with a flow rate of 1 mL/min and a 290 nm detection wavelength (folic acid detection included the use of a fluorescence detector...
set at 311/515 nm excitation emission). The mobile phase increased from 65:35 methanol/water to 70:30 methanol/water over the first 2 min and held there until the method was complete with elution occurring at ~2.2–2.4 min. Peak area under the curve was used to determine the corresponding concentrations of either FA, PEM, or PEM/FA when compared to a standard curve for these mixtures (absorbance for PEM, fluorescence for FA, and absorbance and fluorescence for the PEM/FA mixtures). PEM alone in the PEM/FA mixture could be determined through the difference between the total peak area minus the peak area contributed from FA. This contribution was again determined using a standard curve, which used known mixtures of PEM and FA to determine the peak area associated with the individual PEM and FA contributions. Loaded drug was calculated from the standard curve, which used known mixtures of PEM and FA to determine efficiency was calculated as the ratio of mass in the filtrate to mass in the inlet.

**Cell Viability.** CT26 murine colorectal cancer cells were purchased from ATCC (Manassas, VA). Cells were maintained in RPMI 1640 medium (Corning, Corning, NY) supplemented with 10% FBS (Fisher Scientific, Hampton, NH) and 1% penicillin/streptomycin at 37 °C in an incubator kept at 5% CO2. Cells were washed with PBS, detached using trypsin/EDTA, and diluted to 20,000 cells/mL in a 15 mL conical tube. To plate, 100 μL of the diluted cells (2,000 cells) was added to each well of a 96-well plate and allowed to settle overnight. The next day, the medium was aspirated, and the medium containing the bare NPs or coated NPs was added sequentially in half-fold dilutions to the inner 60 wells of the 96-well plate. Three of the last six wells were left with untreated media as a negative control; the other three of the last six wells were treated with 10% DMSO as a positive control. The cells were returned to the incubator and allowed to grow for 72 h. Following this period, the medium was removed, the cells were washed with PBS, and a new medium was added to each of the wells. A 10 μL solution of the cell viability reagent, Alamar Blue (ThermoFisher, Waltham, MA), was added to each of the inner 60 wells. The plate was returned to the incubator for 90 min and then read at 560/590 nm on a Tecan Infinite plate reader (Tecan, Männedorf, Switzerland). Viability of the cells was calculated as a percentage of the signal detected in each well versus the average signal of the three negative control wells.

**Cell Uptake.** Cells were maintained as described above. To plate, 500,000 cells were seeded into each well of a six-well plate and allowed to settle overnight. The next day, the medium was aspirated, and the medium containing either bare nanoparticles or coated nanoparticles was added to triplicate wells. After 4 h incubation, the cells were washed with PBS and prepared for either microscopy or flow cytometry. For microscopy, cells were fixed with 10% formalin/PBS for 30 min. Following fixation, the cells were washed with PBS and stained with DAPI and Alexa Fluor 488-phalloidin (ThermoFisher, Waltham, MA) for 30 min. After 30 min, the staining solution was aspirated and washed with PBS, and 1 mL of PBS was added to each well. The plate was kept in a 4 °C refrigerator until imaging on an EVOS FL Auto II (ThermoFisher, Waltham, MA).

For flow cytometry, cells were detached from the plate using trypsin/EDTA and diluted with the medium. The cells were spun down to a pellet at 500g for 5 min, and the medium was aspirated and dispersed in PBS. The cells were spun down again at 500g for 5 min, and PBS was aspirated and dispersed in 10% formalin/PBS. The cells were allowed to fix for 30 min. After 30 min, the cells were spun down at 500g for 5 min, and formalin was aspirated and dispersed in PBS. After one final spin at 500g for 5 min, PBS was aspirated, and the cells were dispersed in PBS into 8 μL tubes for flow cytometry (through a filter cap). Samples were assessed using a MACSQuant flow cytometer (Miltenyi Biotec, Cologne, Germany). Fluorescence of no treatment, bare NP-treated, coated NP-treated cells was determined using a 632 nm excitation laser line, with a 655–730 nm emission filter set. Forward scattering/side scattering was used to identify single cells passing through the detector, and 100,000 cells were evaluated in the flow cytometer for each sample to build the distribution.

**In Vivo Treatment Efficacy.** Twenty-five 6 week-old BALB/c mice (Charles River Laboratories, Wilmington, MA) were housed in modified barrier animal facilities prior to tumor inoculation. On the day of tumor inoculation, cell suspensions containing 6 × 106 CT26 cells/mL were prepared in sterile PBS for injection. Fifty microliter injections were made into the right hind flank of isoflurane-anesthetized mice, implanting a total of 3 × 106 cells subcutaneously. The tumors were allowed to grow for 2 weeks to reach an adequate size to begin treatment (>10 mm3, measured twice weekly in this 2 week period by calipers, using the formula 0.5 × long length × short length3).

Once the size threshold had been met, the animals were randomized into four treatment groups. Once randomized, all animals received a 200 μL IV injection (2.1 mg/kg, mass of pemtrexed/mass of animal) of either bare or PEM/FA-NP solution while under isoflurane anesthesia. The infections were repeated once daily for two additional days, for a total of three 200 μL injections. Caliper measurements continued three times per week. Animals were euthanized when meeting one of the following conditions: tumor volume exceeded 2000 mm3, one length measurement by calipers exceeded 20 mm, or ulceration developed superficially on the tumor (weights of the animals were monitored as well, but no animal exhibited weight loss greater than 20% of initial weight to constitute an endpoint by this condition). All animal experiments followed the guidelines set forth by Oregon Health and Science University (OHSU) Institutional Animal Care and Use Committee Protocol #IP00000023.

**Histology.** Organs and tumors were collected from each animal, weighed, and placed into formalin solution (10% by volume in PBS) at 4 °C for 2–3 days. The organs were then transferred to 70% ethanol solution until further processing by the Histopathology Shared Resource at OHSU.

Samples were paraffin embedded, sliced, and prepared according to the required staining protocol, which included either no staining, hematoxylin and eosin (H&E), or caspase-3 (CC3; Promega, 1:1500 dilution). Slides produced from staining were imaged with assistance from the Advanced Light Microscopy Core at OHSU. Scanned images of each slide were taken on a Leica Axios Imaging system (Leica, Wetzlar, Germany) using a 10X objective.

**Statistical Analysis.** All data are represented as mean ± standard deviation. Statistical significance was evaluated using Student’s t-test or logrank (Mantel–Cox) using GraphPad Prism 8 software. P < 0.05 was considered statistically significant, with various asterisks denoting greater confidence (*P < 0.05, **P < 0.01, and ***P < 0.001).
**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01550.

(Figure S1) Characterization of PEM-NP comparison formulation, (Figure S2) in vitro data for the PEM-NPs, (Figure S3) in vivo trends for the PEM-NPs, (Figure S4) images of tumor slices from representative tumors, and (Figure S5) individual animal weights from each of the treatment groups (PDF)

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**Author Contributions**

J.G.R. and C.S. designed the studies. J.G.R., A.N.D., and M.R.L. carried out the studies. J.G.R. and C.S. analyzed the data. J.G.R. and C.S. wrote and edited the manuscript.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

PEM, pemetrexed; IV, intravenous; LbL, layer-by-layer; PE, polyelectrolyte; CML, carboxylate-modified polystyrene latex; PLL, poly-L-lysine; FA, folic acid; PBS, phosphate-buffered saline; TFF, tangential flow filtration; DAPI, 4′,6-diamidino-2-phenylindole; TEM, transmission electron microscopy; MWCO, molecular weight cutoff; HPLC, high-performance liquid chromatography; PES, polyethersulfone; H&E, hematoxylin and eosin; CC3, cleave caspase-3; CRC, colorectal cancer; OHSU, Oregon Health and Science University

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