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Bifunctional Elastin-like Polypeptide Nanoparticles Bind Rapamycin and Integrins and Suppress Tumor Growth in Vivo

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Abstract

Recombinant protein–polymer scaffolds such as elastin-like polypeptides (ELPs) offer drug-delivery opportunities including biocompatibility, monodispersity, and multifunctionality. We recently reported that the fusion of FK-506 binding protein 12 (FKBP) to an ELP nanoparticle (FSI) increases rapamycin (Rapa) solubility, suppresses tumor growth in breast cancer xenografts, and reduces side effects observed with free-drug controls. This new report significantly advances this carrier strategy by demonstrating the coassembly of two different ELP diblock copolymers containing drug-loading and tumor-targeting domains. A new ELP nanoparticle (ISR) was synthesized that includes the canonical integrin-targeting ligand (Arg-Gly-Asp, RGD). FSI and ISR mixed in a 1:1 molar ratio coassemble into bifunctional nanoparticles containing both the FKBP domain for Rapa loading and the RGD ligand for integrin binding. Coassembled

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Notes

The authors declare the following competing financial interest(s): J.P.D., P.S., and J.A.M are inventors on a patent describing the delivery of small molecules using protein polymer fusions related to this work. All other authors declare no competing financial interests.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.7b00469. Figures showing coassembly of copolymers, body weight distribution, microPET images, FSI and ISR/FSI biodistribution, and direct covalent labeling. (PDF)

nanoparticles were evaluated for bifunctionality by performing in vitro cell-binding and drug-retention assays and in vivo MDA-MB-468 breast tumor regression and tumor-accumulation studies. The bifunctional nanoparticle demonstrated superior cell target binding and similar drug retention to FSI; however, it enhanced the formulation potency, such that tumor growth was suppressed at a 3-fold lower dose compared to an untargeted FSI–Rapa control. This data suggests that ELP-mediated scaffolds are useful tools for generating multifunctional nanomedicines with potential activity in cancer.

Graphical Abstract

INTRODUCTION

Breast cancer is the second most common type of cancer, with over 1.3 million newly diagnosed cases worldwide each year.¹ Over the past four decades, chemotherapy has significantly improved breast cancer survival rates from 35% to 77%; however, there remain nearly half a million deaths each year.^{1–4} Cytotoxic chemotherapy remains of primary importance for advanced breast cancer.⁵ Some chemotherapeutics, such as paclitaxel and doxorubicin, benefit from drug carriers to improve the balance between drug toxicity and efficacy.^{6–8} A successful example is paclitaxel preloaded onto albumin, thus called Abraxane. Approved for breast cancer in 2005, it doubled the clinical objective response rate and halved the incidence of severe neutropenia. Beyond Abraxane, next-generation drug carriers offer the opportunity to (i) sequester drug, thereby reducing side effects; (ii) utilize target-mediated delivery and release; (iii) direct multiple modalities to tumors (drugs, proteins, imaging agents); and (iv) facilitate combination drug therapies by mitigating dose-limiting toxicity for one agent.^{9,10} To advance these strategies, our group is developing a new class of recombinant protein-based drug carriers that specifically and noncovalently bind potent small molecules for systemic drug delivery.^{11,12}

Since the approval of Abraxane, newer treatments have targeted molecular subtypes of breast cancer. For example, 20–30% of breast cancer is positive for the HER2 receptor, for which biologics (trastuzumab) and receptor tyrosine kinase inhibitors (lapatinib) have entered the clinic.¹³ Alternatively, more than half of breast cancers are positive for the estrogen or androgen receptors or both,¹⁴ which respond to aromatase therapy (exemestane).

Like chemotherapeutics, drugs such as trastuzumab and exemestane also have dose-limiting side effects such as cardiovascular and endometrial toxicity.^{15,16} Despite progress, there remain significant populations for which these drugs do not work. 12–17% of breast cancer patients are negative for androgen receptor, estrogen receptor, and HER2, and these are defined as triple-negative breast cancer (TNBC).⁵ A large proportion of TNBC overlaps with a group identified by genomic clustering as “basal-like”, which is highly associated with epidermal growth factor receptor (EGFR).^{5,17,18} Due to dependence on EGFR signaling, many TNBCs also require the mammalian target of rapamycin (mTOR) to mediate proliferation.^{17,19} This observation has maintained interest in cytostatic molecules called rapalogues. Like other drugs, rapalogues accumulate in normal tissue, which cause dose-limiting side effects. A pair of Food and Drug Administration approved oral rapalogues, everolimus and rapamycin (Rapa), also have poor bioavailability (15–20%), which may contribute to variable efficacy and toxicity. A clinical trial of everolimus revealed that 75% of breast cancer patients experienced adverse events such as oral sores, pulmonary toxicity, and myelosuppression that necessitated a reduction in dose, and 10% of patients discontinued treatment. Despite these limitations, everolimus was approved to treat hormone-receptor-positive and HER2-negative breast cancer because it doubled progression-free survival.²⁰ Like Abraxane, the rapalogues are logical candidates for advanced drug carriers.

To develop targeted rapalogue carriers for TNBC, our group harnesses protein–polymers (Figure 1).^{11,12,21–24} Protein–polymers are high-molecular-weight polypeptides that can be precisely linked to functional proteins through recombinant protein expression. Among the best-characterized protein–polymers are elastin-like polypeptides (ELPs), which are biologically inspired from human tropoelastin. Composed of the motif (Val-Pro-Gly-*Xaa*-Gly)_{*n*},²⁵ ELP phase separate above a tunable transition temperature that depends on guest residue *Xaa* and length *n*.^{25,26} Phase separation can drive the assembly of ELP nanoparticles.^{27,28} One example is the ELP diblock copolymer SI, which consists of a hydrophilic ELP (Val-Pro- Gly-Ser-Gly)₄₈ connected to a hydrophobic ELP (Val-Pro-Gly-Ile-Gly)₄₈ (Table 1). When heated to physiological temperature, diblock copolymers with the hydrophobic ELP at the carboxy (SI) or amino terminus (IS) assemble ~25 nm radius nanoparticles. As these ELP nanoparticles internalize into cells, they traffic to low-pH compartments enriched in lysosomal proteases, which biodegrades the peptide.²⁹ The SI nanoparticle was modified by fusion to the FK-506 binding protein 12 (FKBP) to generate FSI.^{11,23} FKBP is the intracellular cognate binding partner of Rapa. In the cytosol, FKBP–Rapa complexes are potent inhibitors of mTORC1-mediated proliferation and angiogenic signaling.³⁰ On a drug carrier, FKBP’s high affinity for rapalogues enables them to specifically bind drug for long durations. As such, protein–polymers such as FSI may sequester small molecules during circulation, enhance uptake into important tissues, and shift the toxicity profile of bound drugs.

Among many possible targeting peptides evaluated for cancer, the vitronectin receptor integrin $\alpha_v\beta_3$ has major roles in osteoclast-mediated bone resorption, angiogenesis, pathological neovascularization, and tumor metastasis. Found on many proteins in addition to vitronectin, the Arg-Gly-Asp tripeptide (RGD) binds preferentially to α_v integrin heterodimers, which are up-regulated in endothelial cells recruited by angiogenesis.³¹ This

peptide motif has been widely explored as a targeting ligand on endothelial cells and the TNBC cell line known as MDA-MB-468 ($\alpha_v\beta_5$ positive), which makes it an excellent model ligand with which to explore targeted drug carriers.^{32,33} While these integrins are implicated across a range of cancers, there is clinical evidence that integrin-mediated targeting may have specific utility in treating TNBCs.³⁴

Based on the above observations, this report explores the hypothesis that ELPs can mediate the coassembly of RGD-targeted nanoparticles, thus reducing the dose of FSI-bound Rapa required to suppress tumor growth. A new ELP fusion was synthesized, ISR, in which RGD was genetically fused at the hydrophilic carboxy terminus of a diblock copolymer that contains the same hydrophobic core as FSI. Both ISR and FSI behave similarly to ELP nanoparticles lacking any fusion domains.^{28,35} At low temperatures, these diblock copolymers remain soluble; however, above a critical micelle temperature (CMT), they assemble bifunctional nanoparticles containing both the drug-binding domain (FKBP) and the tumor-targeting ligand (RGD) (Figure 1). Herein, nanoparticles consisting of FSI and ISR are evaluated for their efficacy using in vitro integrin-binding assays, drug retention and cell proliferation experiments, and in vivo tumor regression and accumulation studies. First, the advantage of using FSI for drug binding is confirmed in a xenograft model in comparison to a nanoparticle lacking FKBP (SI). Second, a comparative study was performed at a subtherapeutic dose to demonstrate the relative efficacy of bifunctional nanoparticles compared to FSI-Rapa and free Rapa. Lastly, molecular imaging and confocal microscopy identified bifunctional nanoparticles that accumulate differently in the tumor relative to FSI alone. To the best of our knowledge, this manuscript is the first report showing the in vivo application of coassembly for ELP diblock copolymers with dual functionality. This ELP-mediated assembly strategy may enable the development of new combinations of drugs, imaging agents, or targeting agents onto multifunctional nanostructures with potential applications in cancer, including TNBC.

RESULTS AND DISCUSSION

Coassembly of nanoparticle components provides multi-functionality that might be tailored to load drugs, target receptors, or carry imaging agents. Bifunctional nanoparticles have been reported previously in multiple biomedical applications. For example, gold nanoparticles decorated with positively and negatively charged thiols control cellular uptake.³⁶ Selenium-carbon bifunctional nanoparticles enhance tumor cell death.³⁷ Dendrimer nanovehicles decorated with bifunctional peptides promote neovascular targeting and internalization.³⁸ On the other hand, due to the low aqueous solubility,³⁹ low oral bioavailability,⁴⁰ and adverse side effects such as pulmonary and nephrotoxicity associated with the systemic delivery of Rapa,^{41,42} several advanced materials have been proposed as advanced Rapa carriers in cancer models, such as poly(lactide-*co*-glycolide) nanoparticles,⁴³ polyethylene glycol-*block*-poly(ϵ -caprolactone) nanoparticles,⁴⁴ albumin-bound nanoparticles,⁴⁵ and multidrug-loaded “triolimus” micelles.⁴⁶

Compared with other bifunctional nanoparticles or Rapa carriers evaluated in literature, the nanoparticles in this manuscript are distinctive in two fundamental ways. First, the ELP diblock copolymers are biodegradable polymers generated through recombinant bacterial

expression, which allows the high-fidelity fusion of different peptides such as FKBP and RGD, which bind Rapa and integrins, respectively. Second, these block copolymers are temperature-sensitive and can be tuned to coassemble mixed micelles at physiological temperatures.²⁸ This is supported by a recent report, which confirmed the in vitro coassembly of two ELP diblock copolymers into mixed micelles, whereby one ELP-bound fibrinogen and a second ELP provided temperature-dependent reversal of binding.⁴⁷ To develop the first in vivo evidence that multiple ELP diblock copolymers can work together to improve the delivery of a small molecule, this manuscript describes two ELP diblock copolymer fusions (FSI and ISR) that assemble at physiological temperature into bifunctional nanoparticles (Figure 1).

Characterization and CoAssembly of ELP Diblock Copolymer Nanoparticles

All ELPs in this manuscript were expressed and purified from *E. coli*. After purification, ELPs were characterized for physicochemical properties including purity, temperature–concentration phase diagrams, and particle size (Figure 2). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to confirm the identity and purity of each construct (Figure 2a and Table 1). Optical density was used to study the critical micelle temperature (CMT) of the diblock copolymers, which is inversely related to the logarithm of concentration (Figure 2b). The fusion of FKBP to SI and RGD to IS had minimal influence on the assembly behavior of ELPs. Unlike SI and FSI, the IS and ISR diblock copolymers have positioned a hydrophobic ELP to the amino terminus of the peptide; however, this difference in orientation produces only a small difference (<2 °C) in CMT (Table 1). The addition of the RGD ligand at the carboxy terminus of IS was explored because a prior study by our group demonstrated that such an orientation was successfully able to interact with cell surface integrins.⁴⁸ Nanoparticle size and CMT were further studied using dynamic light scattering (DLS) across the physiological temperature range (Figure 2c). Similar to optical density measurements, the fusion of FKBP to SI and RGD to IS minimally influences particle size. Both FSI and ISR assembled stable nanoparticles above their CMT (25–27 °C) with similar hydrodynamic radii (R_h) of ~25 nm.

After the CMT and the particle size of purified diblock copolymers were studied, the effect of coassembly was evaluated. To determine the optimal mixing ratio of FSI and ISR for coassembly, the CMT and R_h of FSI mixed with ISR in varying ratios was determined (Figure S1). There was no evident change either in the CMT or the hydrodynamic radius with any of the mixtures evaluated. To further support their potential to coassemble bifunctional nanoparticles, FSI and ISR were fluorescently labeled with carboxyfluorescein (CF) and rhodamine (Rho), respectively, mixed in a 1:1 ratio (100 μ M each), and co-localization was studied using confocal microscopy above the CMT (Figure 3a). Confocal images reveal the high spatial co-localization of the two ELP diblock copolymers. Colocalization analysis of green (FSI–CF) and red (ISR–Rho) pixels demonstrated near-perfect colocalization, with a Pearson's coefficient (PC) of 0.976 and an overlap coefficient (OC) of 0.982. A positive slope of the linear regression line (slope = 0.898) and close distribution along the line (r^2 = 0.953) in the pixel plot is consistent with the coassembly of the two ELP diblock copolymers (Figure 3b). Lastly, particle morphology of ISR in the absence and presence of FSI was studied using cryogenic transmission (Cryo-TEM) electron

microscopy (Figure 3c). The particle diameter of ISR was similar to previous reports for FSI¹¹ and was consistent with dynamic light scattering data (Figure 2c). When 50% ISR and 50% FSI mixed nanoparticles were observed, they appeared similar to ISR nanoparticles alone. Coupled with confocal microscopy evidence that FSI–CF and ISR–Rho spatially colocalize, the observation that ISR with and without FSI forms similar-size populations of nanoparticles is consistent with their coassembly into bifunctional nanoparticles (Figure 1).

Recognition of Integrin Receptors by ISR Nanoparticles in Vitro with or without FSI Coassembly

After characterization of the physicochemical and coassembly properties of ELP nanoparticles, the ability of ISR to recognize surface integrin receptors was evaluated on a TNBC cell line (MDA-MB-468) using confocal microscopy (Figure 4). MDA-MB-468 expresses high levels of surface integrin receptors that prove optimal for cell binding.^{32,33} A competition-based integrin-binding assay was performed to study binding of ISR nanoparticles in the presence of c-RGDfK, a cyclic version of RGD peptide known to target and selectively bind surface integrin receptors.^{49,50} ISR was covalently labeled with rhodamine (ISR–Rho) and was competed off the surface using c-RGDfK (Figure 4a). Confocal imaging of 20 μ M ISR–Rho in the absence of c-RGDfK showed high rhodamine staining (panels i–iii), which was significantly decreased when cells were preincubated with c-RGDfK (panels iv–vi). Quantitative image analysis using ImageJ confirmed that c-RGDfK competitively binds to integrin receptors and blocks the cell targeting of ISR presumably due to a higher integrin-binding affinity (Figure 4b). This assay confirmed the specificity of ISR nanoparticles to bind surface receptors, which was further narrowed to the same α_v integrin receptors targeted by c-RGDfK.

After the ability of ISR nanoparticles to bind integrin receptors on MDA-MB-468 cells was confirmed, the effect of coassembly with FSI on target binding was optimized in vitro (Figure 5). ISR was labeled with rhodamine (ISR–Rho), which revealed cellular association in the absence of FSI. Alternatively, FSI was labeled with rhodamine (FSI–Rho), which revealed a lack of cellular association. Similarly, FSI–Rho was mixed in a 1:1 ratio with unlabeled ISR (50% ISR and 50% FSI) to determine if coassembly with unlabeled ISR would enhance cellular association of labeled FSI. ISR–Rho efficiently mediated cell binding as visualized by red fluorescence (Figure 5a panels i–iii), while FSI–Rho alone did not bind (Figure 5a panels iv–vi). Only when mixed with unlabeled ISR did the fluorescence intensity of FSI–Rho elevate (Figure 5a panels vii–ix), which is consistent with coassembly of the two diblock copolymers observed in Figure 3. Fluorescence intensity was quantified using image analysis, and FSI–Rho uptake in the absence and the presence of ISR was compared (Figure 5b). Cells treated with ISR–Rho had significantly more fluorescence compared to FSI–Rho and 50% ISR and 50% FSI each (Tukey's post-hoc analysis, $\alpha = 0.05$, $p < 0.0001$ each). Moreover, cells treated with 50% ISR and 50% FSI also had significantly more fluorescence than FSI–Rho (Tukey's post-hoc analysis, $\alpha = 0.05$, $p < 0.0001$). To further quantify the relationship between the mixing ratio of coassembly and the cell targeting efficiency, flow cytometry was performed. A clear trend was observed in which increasing percentages of unlabeled ISR enhanced the number of cells positive for FSI–Rho (Figure 5c). Bifunctional nanoparticles coassembled from 50% ISR and 50% FSI yielded

~90% positive cells, which was equivalent to that obtained by cell incubation with the positive control of 100% ISR–Rho alone. FSI–CF and ISR–Rho co-localization observation (Figure 3) coupled with integrin binding on MDA-MB-468 cells (Figure 4, 5) suggests that coassembly with FSI into bifunctional nanoparticles did not affect the ability of ISR to bind cell surface integrins.

Bifunctional Nanoparticle Demonstration of Stable Drug Loading and Retention

One advantage of the bifunctional nanoparticles is that by adjusting the mixing ratio of FSI and ISR diblock copolymers, the functionality of the coassembled nanoparticle can be optimized for either drug delivery or tumor targeting. However, because 50% ISR and 50% FSI nanoparticles efficiently targeted 90% of cancer cells (Figure 5c), the 1:1 ratio was selected to evaluate drug loading, stability, and retention (Figure 6). ISR alone was included as a negative control to account for nonspecific solubilization of the drug in the absence of FKBP. For bifunctional ISR/FSI nanoparticles, FSI and ISR were premixed in a 1:1 ratio before Rapa loading to measure the ratio of drug binding to total ELP. At 0 h after drug loading, the Rapa-to-ELP ratio was 0.77 ± 0.04 for FSI compared to 0.60 ± 0.01 for ISR/FSI bifunctional nanoparticles, reflecting the reduced number of FKBP domains in the formulation available to bind Rapa (Figure 6a). The moderate Rapa-to-ELP ratio of 0.29 ± 0.02 for ISR alone was attributed to nonspecific binding of hydrophobic Rapa into the hydrophobic core of ISR nanoparticles. After drug loading, the stability of each formulation was evaluated using DLS by measuring the R_h at 37 °C (Figure 6b). The R_h values of FSI and bifunctional ISR/FSI nanoparticles loaded with Rapa remained stable for at least 24 h. Moreover, the R_h values before and after Rapa loading were similar, which confirms that noncovalent drug association with the nanoparticles has no effect on their size or stability. After the quantification of loading and stability, the ability of each formulation to retain Rapa was examined using dialysis under sink conditions (Figure 6c). Poor drug retention was observed for ISR, which was expected due to its lack of drug-binding FKBP domains. In contrast, there was strong drug retention observed for FSI, which is consistent with our previous reports;¹¹ furthermore, similar retention was observed for the bifunctional ISR/FSI nanoparticles. Both FSI and ISR/FSI showed similar two-phase release kinetics compared to a one-phase fast release observed with ISR. This suggests that coassembly had little influence on Rapa release compared to that of FSI alone.

FSI–Rapa and ISR/FSI–Rapa Potency in Inhibiting Cell Proliferation Compared to Free Rapa in Vitro

Having confirmed the ability of bifunctional ISR/FSI nanoparticles to recognize and bind integrin receptors and retain Rapa during dialysis, we next studied their ability to deliver Rapa intracellularly by assessing the in vitro cytostatic efficacy of FSI–Rapa, ISR/FSI–Rapa, and free Rapa control on MDA-MB-468 cells. All of the three groups inhibited cell proliferation in a dose-dependent manner (Figure 7a). There was no detectable difference between FSI–Rapa and ISR/FSI–Rapa, which suggested that ability of ISR to bind integrin receptors at low concentrations (0–100 nM) does not play a distinguishing role in the cellular accessibility of Rapa in vitro. Instead, both of the formulations appear capable of delivering drug to the cytosol, where they are expected to inhibit proliferation through intracellular mTORC1.⁵¹ Interestingly, both the FSI and the ISR/FSI formulations were

significantly more potent than free Rapa. The concentration corresponding to a 50% suppression in proliferation, IC_{50} , for FSI-Rapa, ISR/FSI-Rapa, and free Rapa were found to be 0.21 ± 0.02 , 0.32 ± 0.16 , and 1.81 ± 0.96 nM, respectively ($n = 3-4$, mean $\pm$ SD). Tukey's post-hoc analysis revealed statistically significant differences between free Rapa and FSI-Rapa and between free Rapa and ISR/FSI-Rapa (Figure 7b, $\alpha = 0.05$, $p = 0.0004$ and 0.001 , respectively). This observation was consistent with our recent report, in which free Rapa was found to be significantly less potent in inhibiting MDA-MB-468 growth in vitro in comparison to a library of structurally related FKBP-ELP carriers.¹² Because Rapa binds strongly to serum albumin (>95%),⁵² if the FSI or ISR/FSI formulations were to rapidly release Rapa during incubation, the liberated drug might be expected to bind the great excess of bovine albumin in the medium and one would thus predict a similar IC_{50} as that observed for the free drug. Instead, this downward shift in the IC_{50} with respect to free Rapa suggests that both the FSI and the ISR/FSI formulations maintain their interaction with Rapa, even in complete medium.

Bifunctional Nanoparticles Suppression of Tumor Growth in Vivo at a 3-Fold Lower Dose than Untargeted Nanoparticles

After optimization of the bifunctional ISR/FSI formulation for in vitro integrin binding, drug retention, and efficacy, the coassembled nanoparticles were next tested for in vivo bifunctionality using human MDA-MB-468 orthotopic breast cancer xenografts (Figure 8). A pair of tumor-regression studies were performed. The first study was designed to confirm the advantage of using FKBP-decorated nanoparticles (FSI) for systemic delivery of Rapa in comparison with ELP nanoparticles lacking FKBP (SI) and free Rapa controls and to estimate an intermediate subtherapeutic dose for the untargeted FSI formulation (Figure 8a). As can be seen in Figure 6c and observed previously,¹¹ FSI nanoparticles loaded with excess Rapa also solubilize some weakly associated drug into the hydrophobic core, which leaves the formulation under dialysis with a ~2 h half-life. Under the rationale that integrin targeting may work best if the drug is strongly retained by the formulation during circulation, any weakly associated drug was dialyzed away from FSI formulations for 12 h (6 half-lives) prior to in vivo administration. These dialyzed FSI-Rapa formulations contain only strongly associated Rapa that is tightly bound to FKBP. This formulation was compared at an equivalent dose of 0.25 mg Rapa/kg BW to free Rapa solubilized either in DMSO or in SI nanoparticles lacking the FKBP domain. The formulations were injected intravenously via the tail vein. The results reveal superior antitumor efficacy of FSI-Rapa compared with either SI-Rapa or free Rapa controls (Figure 8a). The tumor suppression with FSI-Rapa at 0.25 mg/kg was statistically significant compared to phosphate-buffered saline (PBS) (Tukey's post-hoc analysis, $\alpha = 0.05$, $p = 0.001$). These results confirm the importance of using the FKBP domain for effective systemic delivery of Rapa. The same study also examined three lower doses of FSI-Rapa at 0.0075, 0.025, and 0.075 mg/kg Rapa dose to identify an intermediate sub-therapeutic dose. A decreasing trend of antitumor efficacy was observed for corresponding doses; furthermore, FSI-Rapa at 0.075 mg/kg appeared to be the highest dose with a subtherapeutic response (Figure 8a). Based on this observation, the dose of 0.075 mg/kg was selected for further evaluation of the bifunctional nanoparticles relative to FSI-Rapa. To accomplish this, a second tumor-regression study was then performed comparing four groups: (i) PBS; (ii) free Rapa; (iii) FSI-Rapa; and (iv) ISR/FSI-Rapa at the

equivalent dose of 0.075 mg/kg (Figure 8b). Groups treated with free Rapa showed weak tumor suppression compared to the intermediate response observed with FSI–Rapa treatment. The FSI–Rapa group alone was not significantly different than either PBS or free Rapa groups. Encouragingly, only the bifunctional formulation ISR/FSI–Rapa significantly inhibited tumor volume compared with PBS and free Rapa treatments (Tukey’s post-hoc analysis, $\alpha = 0.05$, $p = 0.003$ and 0.02 , respectively). Kaplan–Meier survival analysis was employed to distinguish the time by which this slow-growing tumor model exceeded the initial tumor volume, which was defined as the mean (69 mm^3) plus 4 times the standard deviation (22 mm^3) of tumor volumes across all tumors on the first day of the study. Based on a normal distribution of this population, only 1 in $\sim 10\,000$ tumors would randomly fall above $\sim 150 \text{ mm}^3$. Thus, tumors that exceeded this threshold were considered to have reached their end point. This threshold revealed 100% “survival” for mice treated with ISR/FSI–Rapa compared with other formulations (Figure 8c). The four treatment groups were compared by six pair-wise log-rank tests using a Bonferroni-corrected $\alpha = 0.05/6 = 0.008$ as the criteria for significance. Treatment with FSI–Rapa significantly prolonged survival compared to PBS ($\alpha = 0.008$, $p = 0.005$). The bifunctional ISR/FSI–Rapa treatment further extended survival compared with PBS, free Rapa, and FSI–Rapa ($\alpha = 0.008$; $p = 0.001$, 0.001 , and 0.002 , respectively). Orthotopic MDA-MB-468 xenografts have previously been treated with Rapa at high doses of 3–15 mg/kg or in combination with drugs such as paclitaxel or lapatinib to achieve tumor regression.^{53,54} Compared to these reports, the untargeted FSI–Rapa formulation delays tumor growth at an impressively low dose of 0.25 mg/kg dose. Furthermore, coassembly with ISR into a bifunctional nanoparticle further reduced the efficacious dose by at least 3-fold to 0.075 mg/kg, presumably due to binding of ISR to cell-surface integrins. At these low doses of Rapa, all treatments were well-tolerated; furthermore, no changes in body weight were observed (Figure S2).

Bifunctional Nanoparticle Exhibition of Higher Tumor Accumulation Compared with Untargeted Nanoparticles

To explore whether an enhancement in tumor uptake was associated with the enhancement in observed activity of ISR/FSI (Figure 8b), the biodistribution and tumor micro-distribution of the bifunctional nanoparticle was compared to FSI alone in the same xenograft model. The tumor-targeting efficacy of the bifunctional nanoparticle was evaluated using a microPET imaging study (Figure S3) and confocal microscopy on excised tumor sections (Figure 9). Previously, an ELP nanoparticle was observed to have a blood half-life of $\sim 4 \text{ h}$ when administered intravenously in an orthotopic tumor mouse model.⁵⁵ To independently determine the pharmacokinetics and tumor biodistribution of FSI and of ISR/FSI, new molecular imaging studies were performed (Figures S3 and S4). By tracking the intensity of ^{64}Cu -labeled ISR/FSI and FSI in the heart, which has a signal dominated by free blood, estimates for pharmacokinetic parameters were determined (Table 2). ISR/FSI had a half-life of $1.1 \pm 0.2 \text{ h}$ ($n = 8$), which was similar to $1.0 \pm 0.3 \text{ h}$ ($n = 11$) for FSI. Likewise, ISR/FSI and FSI had similar blood clearance values of $8.2 \pm 4.4 \text{ mL/h}$ ($n = 8$) and $5.3 \pm 3.5 \text{ mL/h}$ ($n = 11$), respectively. To determine if there was a significant difference in tumor biodistribution, tumor accumulation (% ID/g) was obtained by drawing a region of interest (ROI) over tumors at 8 h. The tumor concentration for ISR/FSI was found to be $4.9 \pm 3.1\%$ ID/g ($n = 8$), which was significantly higher than $2.4 \pm 1.8\%$ ID/g ($n = 11$) for FSI (t test, α

= 0.05, $p = 0.04$). Thus, the bifunctional nanoparticle appears to moderately, but detectably, increase overall biodistribution to the tumor.

To further explore the tumor microdistribution of both of the carriers, in vivo tracking of the nanoparticles was next studied using confocal microscopy of rhodamine-labeled SI, which was efficiently labeled at its single amino terminus and coassembled with unlabeled FSI or ISR/FSI nanoparticles. These assemblies remained stable colloids for at least 12 h at 37 °C (Figure S5). Direct modification of FSI with rhodamine was possible for short-duration in vitro studies (Figure 5); however, rhodamine conjugation shortened the stability of FSI nanoparticles at 37 °C, making direct labeling challenging to interpret over longer in vivo studies (Figure S5). Equivalent doses of rhodamine were administered via the tail vein for each formulation to mice carrying the MDA-MB-468 tumors ($n = 3$ per group). A third PBS-treated group was evaluated to establish the background fluorescence in tumor sections. Based on the pharmacokinetics and blood half-lives obtained from the microPET study, as well as the stability of the rhodamine-labeled formulation, tumors were obtained 2 and 8 h post-injection. A total of 8 h accounts for ~8 half-lives, by which the majority of accumulation was expected. Tumors were flash-frozen in OCT and cryo-sections were observed under confocal microscopy for rhodamine fluorescence. There was no rhodamine fluorescence above PBS background observed at 2 h. After 8 h, the rhodamine fluorescence of FSI and ISR/FSI was above background levels, but only ISR/FSI nanoparticles showed distinct and significant tumor accumulation (Figure 9a). The normalized fluorescence of bifunctional nanoparticles observed in tissue sections at 8 h ($n = 6$ –12 per group, mean $\pm 95\%$ CI) was significantly greater compared with PBS, FSI at 2 h, and ISR/FSI at 2 h each (Tukey's post-hoc analysis; $\alpha = 0.05$; $p = 0.013$, 0.004, and 0.001, respectively) (Figure 9b). Using this confocal imaging, it was not possible to detect a significant difference between FSI and ISR/FSI in the tumor at 8 h. What is clear from these confocal images is that the intratumoral accumulation of ISR/FSI appears to be enhanced in a small population of cells within the excised tumor (Figure 9a). This observation is consistent with the significantly higher tumor accumulation observed for ISR/FSI in the biodistribution study (Figure S3) and the significantly enhanced efficacy of ISR/FSI at a low dose of 0.075 mg/kg (Figure 8b,c). The $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been reported to play a role in angiogenesis and tumor metastasis;⁵⁶ selective targeting of these receptors by cyclic RGD peptides have been reported to inhibit angiogenesis in vitro.⁵⁷ If the population of cells that accumulate ISR/FSI is involved in angiogenesis, RGD-mediated shifts in the intracellular delivery of Rapa could explain how the bifunctional nanoparticles are effective at a 3-fold lower dose of 0.075 mg/kg in contrast to FSI alone.

CONCLUSIONS

This report provides the first in vivo application of bifunctional ELP nanoparticles that can carry potent payloads and accumulate in solid tumors. ELP diblock copolymers tagged with two functional cargos were shown to coassemble nanoparticles that either bind Rapa or integrins on solid tumors. Coassembly had minimal influence on the particle size or morphology of ELP nanoparticles, which efficiently targeted MDA-MB-468 breast cancer cell line in vitro. The ability of FSI to bind and retain Rapa was not affected by coassembly with ISR, which suggests that the formulation may retain the drug in vivo. These

bifunctional nanoparticles suppressed tumor growth in a xenograft model at a 3-fold lower dose compared to FSI–Rapa nanoparticles alone. When tumor accumulation was studied using confocal microscopy, a subpopulation of fluorescence-positive cells were observed relative to FSI nanoparticles, which was consistent with the higher tumor accumulation of bifunctional nanoparticles observed using microPET imaging. These results are consistent with in vitro data showing that these carriers target cells known to uptake the RGD tripeptide via heterodimeric integrins. While additional optimization remains, this modular coassembly strategy may facilitate the design of better multifunctional nanomedicines carrying various cargos, including imaging agents, drugs, and targeting ligands.

EXPERIMENTAL PROCEDURES

ELP Gene Design and Purification

Cloning of FKBP to ELP⁵⁸ and RGD to ELP⁴⁸ was performed as described previously. pET25b (+) vectors encoding ELP genes were transfected into BLR (DE3) *E. coli* cells (69053, EMD Millipore, Billerica, MA) and plated on agar with 100 $\mu\text{g/mL}$ ampicillin. A single colony for each construct was inoculated in 50 mL of autoclaved Terrific broth media (12105, Mo Bio Laboratories, Carlsbad, CA) supplemented with 100 $\mu\text{g/mL}$ ampicillin and grown overnight at 37 °C. Starter cultures were then amplified in 3–4 L batches supplemented with 100 $\mu\text{g/mL}$ ampicillin and allowed to grow for 24 h at 37 °C. Cell lysis and ELP purification was performed using inverse transition cycling (ITC), as described previously.^{11,35} Purified protein in sterile PBS was filtered using 200 nm sterile Acrodisc 25 mm filters (PN 4612, Pall Corporation, Port Washington, NY) and assayed for concentration using Beer–Lambert's law:

$$\text{protein concentration } (M) = \frac{(A_{280} - A_{350}) \times \text{dilution factor}}{\text{MEC} \times l} \quad (1)$$

where M is molar concentration; A_{280} and A_{350} are absorbance at 280 and 350 nm; respectively; l is the path length (cm); and MEC is the estimated molar extinction coefficient at 280 nm: 1285 $\text{M}^{-1} \text{cm}^{-1}$ for SI, IS, and ISR; and 11585 $\text{M}^{-1} \text{cm}^{-1}$ for FSI.⁵⁹ Purified yields were ~50 mg/L of bacterial culture.

ELP Physicochemical Characterization

ELP purity was determined using SDS-PAGE gel analysis. A total of 10 μg of protein was mixed with SDS Laemmli loading buffer (1610747, Bio-Rad, Hercules, CA) containing 10% v/v β -mercaptoethanol, heated at 90 °C for 5 min and loaded onto 4–20% gradient Tris–glycine–SDS-PAGE gel (58505, Lonza, Walkersville, MD). The gel was stained using 10% w/v copper chloride and imaged using a ChemiDoc Touch Imaging system (Bio-Rad; Figure 2a). Peak areas in each lane were calculated using ImageJ (National Institutes of Health, Bethesda, MD), and purity was estimated using the following equation:

$$\text{percent purity} = \frac{A_{\text{peak}}}{A_{\text{tot}}} \times 100 \quad (2)$$

where A_{peak} is the area of the protein band peak, and A_{tot} is the total area under all the peaks per lane. The ELP critical micelle temperature was determined using a DU800 UV–vis spectrophotometer (Beckman Coulter) by performing temperature ramps at 1 °C/min and measuring OD at 350 nm. The temperature at the maximum first derivative of OD 350 nm was defined as the CMT (Figure 2b). DLS was utilized to estimate the hydrodynamic radii of ELP nanoparticles (Figure 2c). ELP samples (25 μM) in PBS were filtered at 4 °C (Whatman Anotop 0.02 μm , Sigma-Aldrich, St. Louis, MO) and monitored from 18 to 37 °C at the rate of 1 °C/min on a Wyatt Dynapro plate reader (Santa Barbara, CA) in a pre-chilled 384 black well plate.

Coassembly of ELP Diblock Copolymers

FSI and ISR coassembly was studied using confocal laser scanning microscopy, cryogenic transmission electron microscopy, optical density, and dynamic light scattering. FSI and ISR samples were covalently labeled with carboxyfluorescein or rhodamine, respectively, using *N*-hydroxysuccinimide chemistry. FSI–CF and ISR–Rho were mixed (100 μM each) on a 35 mm glass-bottom dish (MatTek, MA) and imaged at 30 °C using a Zeiss LSM 510 Meta NLO confocal microscopy (Thornwood, NY) with an Instec HCS60 temperature control stage (Denver, CO; Figure 3a). The JACoP plugin in ImageJ (NIH) was used to determine Pearson's coefficients and overlap coefficients.⁶⁰ The slope and r^2 were used to correlate the green and red pixel intensities (Figure 3b), as described previously.³⁵ Cryo-TEM was performed to observe bifunctional nanoparticles in solution (Figure 3c), as described previously.¹¹ FSI and ISR coassembly at different mixing ratios was further characterized using optical density measurements and DLS (Figure S1).

Integrin-Mediated Binding and Flow-Cytometry Assays

MDA-MB-468 cells (HTB-132, American Type Tissue Culture Collection, Manassas, VA) were cultured at 37 °C with 5% CO₂ in Dulbecco's modified Eagle medium and F-12 media (DFL21, Caisson laboratories) supplemented with 10% v/v heat inactivated fetal bovine serum (FBS, 35-011-CV, Corning). Cells (3×10^5) were first blocked for 1 h at 37 °C with either PBS or 125 μM c-RGDfK followed by the addition of 20 μM ISR–Rho. Cells were incubated for 1 h at 37 °C, fixed with 4% paraformaldehyde in PBS, treated with 4',6-diamidino-2-phenylindole for nuclear staining, and imaged using a Zeiss LSM 510 Meta NLO confocal microscope (Thornwood, NY) (Figure 4a). All images were analyzed by ImageJ software to calculate an area-normalized fluorescence measurement from the pixel intensities (Figure 4b) using the following equation:

$$\text{normalized fluorescence} = \frac{I_{\text{Rho}}}{A_{\text{tot}}} \quad (3)$$

where I_{Rho} is the integrated density of rhodamine fluorescence, and A_{tot} is the total area per image ($n = 3$, mean $\pm$ SD).

The effect of coassembly on cell binding was evaluated on MDA-MB-468 cells (Figure 5). A total of three samples were labeled with rhodamine: (i) ISR–Rho; (ii) FSI–Rho; and (iii)

50% FSI–Rho mixed with 50% unlabeled ISR (50% ISR and 50% FSI). Cells (3×10^5) were incubated with 200 μM ELP for 1 h at 37 °C, fixed with 4% paraformaldehyde in PBS, treated with DAPI for nuclear staining, and imaged using confocal microscopy (Figure 5a). ImageJ was used to quantify pixel intensity using eq 3. The normalized fluorescence ($n = 3$, mean $\pm$ SD) across 3 groups was compared using one-way ANOVA ($\alpha = 0.05$, $p < 0.0001$). Tukey–Kramer post-hoc analysis was performed to test significance between the individual groups as reported in the results (Figure 5b). For flow cytometry, cells (1×10^4) were treated with the mixtures of FSI–Rho and unlabeled ISR (5% to 50%) for 1 h. 100% FSI–Rho and 100% ISR–Rho were used as negative and positive controls, respectively. The cells were washed, resuspended in 500 μL of sterile PBS, and analyzed on an Attune acoustic focusing cytometer (Life Technologies). Data was analyzed using FlowJo (FlowJo LLC, Ashland, OR). Negative cells were gated for baseline fluorescence, and cells above the gate were considered positive for association of ISR/FSI–Rho mixtures (Figure 5c).

Rapa Loading and Retention by Dialysis and Formulation Preparation for in Vivo Administration

A two-phase solvent evaporation method was used to load Rapa (LC laboratories, Woburn, MA). Briefly, 250 μM total ELP concentration dissolved in PBS (FSI, ISR, or FSI and ISR mixed in a 1:1 ratio) was equilibrated in a glass vial at 35 °C for 10 min followed by the addition of 250 μM Rapa dissolved in hexane to obtain a two-phase mixture of ELP (aqueous phase) at the bottom and Rapa (organic phase) at the top. The organic phase was evaporated under mild flow of dry N_2 gas with continuous stirring on a magnetic stir plate over 10–15 min, and the remaining aqueous solution was centrifuged (13 000 rpm, 10 min, 35 °C) to remove free drug precipitate. The supernatant containing ELP was then evaluated for Rapa concentration at 280 nm using C-18 reverse-phase high-performance liquid chromatography (RP-HPLC) column (186003033, Waters, Milford, MA). RP-HPLC runs were performed using mobile phase A (water with 0.1% TFA) and phase B (methanol with 0.1% TFA) over a gradient from 40% to 95% of phase B. Unknown sample concentrations were determined from the area under the curve for a Rapa peak using a calibration standard. After quantification, the Rapa-to-ELP ratio was plotted as an indicator of Rapa loading (Figure 6a). ELP nanoparticles with and without drug loading were evaluated for stability by measuring R_h at 37 °C ($n = 3$, mean $\pm$ SD) using DLS for a period of 24 h (Figure 6b). Rapa-loaded ELPs were then dialyzed against water under sink conditions for an equivalent period to determine drug retention. Aliquots were withdrawn at fixed time intervals and analyzed for Rapa concentrations using RP-HPLC as explained above. Nonlinear regression analysis was employed to fit the retention of Rapa in FSI, ISR, and bifunctional ISR/FSI formulations over time (Figure 6c).

For in vivo administration, FSI–Rapa was dialyzed after drug loading for 12 h to remove any weakly associated drug from the hydrophobic core. FSI–Rapa alone or mixed with ISR in a 1:1 ratio was diluted in sterile PBS to Rapa concentrations required for the indicated dosage (mg/kg BW) and frozen at -80 °C as aliquots for single use. Aliquots were thawed at 4 °C, heated above the CMT for 10 min at 35 °C to initiate the assembly of FSI–Rapa nanoparticles or the coassembly of bifunctional ISR/ FSI–Rapa nanoparticles, and injected intravenously via the tail vein.

Cell Proliferation Assay

A formazan-based cell proliferation assay was performed using the MDA-MB-468 cells as described previously.¹² Briefly, 3,000 cells in 100 μ L of media per well were seeded in 96 wells in triplicate. Cells were allowed to adhere for 24 h, and media was replaced with fresh media followed by treatment with dilutions of complete media containing free Rapa, FSI-Rapa, or ISR/FSI-Rapa. After 72 h, the WST-1 reagent was incubated with the cells for 3 h according to the manufacturers recommendations (11644807001, Sigma-Aldrich, St. Louis, MO). Tetrazolium conversion to formazan was monitored by absorbance at 440 nm after subtracting for a reference wavelength at 690 nm. The percent maximal proliferation was calculated and plotted against Rapa concentration (Figure 7a) using the following equation:

$$\text{maximal proliferation (\%)} = \frac{(A_{\text{treated}} - A_0)}{(A_{\text{untreated}} - A_0)} \times 100 \quad (4)$$

where A_{treated} is the corrected absorbance of cells treated with Rapa, $A_{\text{untreated}}$ is the corrected absorbance of untreated cells, and A_0 is the corrected absorbance of media without cells. The IC_{50} was determined using nonlinear regression in independent assays ($n = 3$ –4, mean $\pm$ SD). A global one-way ANOVA on the $\log_{10}$ -transformed IC_{50} revealed significant differences among the three Rapa-treatment groups ($\alpha = 0.05$, $p = 0.0004$). Tukey–Kramer post-hoc analysis was then performed to test significance between the individual groups as reported in the results (Figure 7b).

Tumor-Regression Studies

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Southern California. A single injection of MDA-MB-468 cells in serum-free media ($\sim 2 \times 10^6$) was implanted into the mammary fat pad of 7-week-old female athymic nude mice (Nude-*Foxn1^{nu}*, Envigo Inc., Indianapolis, IN). Orthotopic tumors were allowed to grow to an average volume between 50 and 100 mm^3 , and mice were randomly assigned into groups before start of the treatment. A total of 100 μ L of each formulation was injected three times a week through the tail vein. Body weight and tumor size were recorded three times a week. Tumor size was measured using an electronic caliper, and the tumor volume was calculated using the following equation:

$$\text{tumor volume (mm}^3\text{)} = \frac{a^2 \times b \times \pi}{6} \quad (5)$$

where a and b represent shorter and longer tumor length, respectively. A pair of tumor studies were evaluated. The first study compared seven groups: (i) PBS ($n = 6$); (ii) SI-Rapa, 0.25 mg/kg ($n = 6$); (iii) free Rapa in DMSO, 0.25 mg/kg ($n = 7$); (iv) FSI-Rapa, 0.25 mg/kg ($n = 6$); (v) FSI-Rapa, 0.0075 mg/kg ($n = 3$); (vi) FSI-Rapa, 0.025 mg/kg ($n = 3$); and (vii) FSI-Rapa, 0.075 mg/kg ($n = 3$) (Figure 8a). Tumor volumes on the last day of treatment had significant differences by a global one-way ANOVA ($\alpha = 0.05$, $p = 0.006$). Tukey–Kramer post-hoc analysis was performed to test significance between the individual groups, as

reported in the results. The second study compared four groups ($n = 5$ – 6 per group): (i) PBS; (ii) free Rapa in DMSO; (iii) FSI–Rapa; and (iv) ISR/FSI–Rapa at an equivalent dose of 0.075 mg/kg (Figure 8b). Tumor volumes on the last day of the treatment had significant differences by a one-way ANOVA ($\alpha = 0.05$, $p = 0.004$). Following ANOVA, Tukey–Kramer post-hoc analysis was performed to test significance between the individual groups as reported in the results. For Kaplan–Meier survival analysis, tumor volume reaching 150 mm³ were defined as the end-point (Figure 8c). The log-rank test (Mantel–Cox) revealed significant differences between survival curves ($\alpha = 0.05$ with 95% CI, $p = 0.0001$). Individual treatment groups were compared pair-wise to the PBS treatment group using a Bonferroni correction ($\alpha = 0.05/6$ comparisons = 0.008) as reported in the results. Body weights for the studies are shown as mean $\pm$ SD (Figure S2).

Chelation and ⁶⁴Cu Radiolabeling

⁶⁴Cu radiolabeling was performed using AmBaSar-conjugated proteins. AmBaSar was activated by 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide hydrochloride and *N*-hydroxysulfosuccinimide at pH 4.0 for 30 min (4°C) with a molar ratio of AmBaSar to EDC to SNHS of 10:9:8. Then, 5-fold molar excess of activated AmBaSar was mixed with backbone ELP, SI in borate buffer (0.1 M, pH 8.5) and reacted overnight at 4°C . Size-exclusion PD-10 chromatography was used to remove unreacted AmBaSar. The AmBaSar-conjugated SI protein was labeled with ⁶⁴Cu by addition of 1 mCi of ⁶⁴Cu (10 nmol protein per mCi ⁶⁴Cu) in 0.1 M ammonium acetate (pH 5.5) buffer followed by 45 min of incubation at 40°C . Before purifying ⁶⁴Cu–SI using a PD-10 column, diethylenetriaminepentaacetic acid (3 μL , 10 mM, pH 6.02) was added to remove ⁶⁴Cu that weakly interacts with the peptide backbone.

MicroPET Imaging

Molecular imaging was performed using a microPET R4 rodent model scanner (Concorde Microsystems, Knoxville, TN). As described above, nude mice carrying ~ 100 mm³ orthotopic MDA-MB-468 tumors in the mammary fat pad were anesthetized with 2% v/v isoflurane gas with oxygen followed by intravenous injection of (i) ~ 116 μCi ⁶⁴Cu–SI/FSI ($n = 11$) and (ii) ~ 128 μCi ⁶⁴Cu–SI/ISR/FSI ($n = 8$). Static scans were obtained at 5 min, 1, 2, 4, and 8 h post-injection (Figure S3). The images were reconstructed by a two-dimensional ordered subset expectation maximum algorithm. Time-activity biodistribution in selected tissues were obtained from the PET scans by drawing regions of interest (ROI) over the tissue area using the AMIDE software package (<http://sourceforge.net>). The counts per pixel per minute obtained from the ROI were converted to counts per milliliter per minute using a calibration constant obtained from scanning a cylinder phantom. The ROI counts per milliliter per minute were converted to counts per gram per minute, assuming a tissue density of 1 g/mL, and divided by the injected dose to obtain a percent injected dose of ⁶⁴Cu tracer retained per gram of tissue (% ID/g). This method was used to plot concentration at each PET scan time point for all the tissues (Figure S4). Signal intensity in the heart decreased in a log–linear manner over the first 2 h, which was used to estimate pharmacokinetic half-life and clearance for both formulations (Table 2).

Confocal Microscopy of MDA-MB-468 Solid Tumors

Evidence for differences between intratumoral uptake of bifunctional ISR/FSI relative to FSI nanoparticles was determined using confocal microscopy on excised tumors (Figure 9). The backbone ELP, SI, was covalently labeled with rhodamine using NHS chemistry (labeling efficiency of ~15%) and coassembled with either (i) FSI or (ii) ISR/FSI for 10 min at 37 °C prior to intravenous administration. Mice ($n = 3$ per group) were injected by tail vein with (i) PBS, (ii) 0.15 mg of Rho per kilogram of SI-Rho/FSI; and (iii) 0.15 mg of Rho per kilogram of SI-Rho/ISR/FSI. After injection, mice from the SI-Rho/FSI and the SI-Rho/ISR/FSI groups were euthanized, and tumors were excised at 2 and 8 h each. Tumors from the PBS group were excised to study background fluorescence. Tumors were flash-frozen on dry ice using optimum cutting temperature solution (4583, VWR, Radnor, PA) in Tissue-Tek cryomolds (4557, VWR) and preserved at -80°C . Tumors were cry-sectioned ($5\ \mu\text{m}$ thickness) and mounted on glass slides. Samples were incubated with NucBlue Live ReadyProbes Reagent (R37605, Thermo Fisher Scientific, Waltham, MA) for 20 min, mounted with ProLong antifade mounting medium and protected from light overnight before imaging by a ZEISS LSM 800 confocal laser scanning microscope (Figure 9a). Images were analyzed by ImageJ to determine rhodamine pixel intensity using eq 3 and plotted as mean $\pm 95\%$ CI ($n = 6\text{--}12$ sections per group) (Figure 9b). Fluorescence at 8 h differed significantly using a global one-way ANOVA between all three groups ($\alpha = 0.05$, $p = 0.0007$). Tukey–Kramer post-hoc analysis was performed to test significance between individual groups, as reported in the results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Rapa	rapamycin
ELP	elastin-like polypeptides
FKBP	FK-506 binding protein 12
SI	S48I48
IS	I48S48
FSI	FKBP-S48I48
ISR	I48S48-RGD

ITC	inverse transition cycling
CMT	critical micelle temperature
R_h	hydrodynamic radius
MW	molecular weight
TNBC	triple negative breast cancer
mTORC1	mammalian target of rapamycin complex 1
DLS	dynamic light scattering
TEM	transmission electron microscopy
RP-HPLC	reverse-phase high-performance liquid chromatography
PET	positron emission tomography
ROI	region of interest

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Figure 1.

Coassembly of bifunctional ELP nanoparticles with specificity of drug and receptor binding. Obtained from recombinant cellular expression, ELPs are a useful platform with which to engineer precision macromolecules for drug delivery. For example, the ELP diblock copolymer known as FSI assembles nanoparticles when heated above its critical micelle temperature (CMT); furthermore, these nanoparticles are decorated with a small protein (FKBP) that binds a potent cytostatic small molecule (Rapa). To examine the hypothesis that mixtures of ELP diblock copolymers can coassemble bifunctional nanoparticles, this manuscript explores triggered assembly of FSI with a second ELP called ISR. ISR contains the RGD ligand, which binds cells expressing heterodimeric integrins in tumors. When mixed and heated to physiological temperature, ISR and FSI assemble bifunctional nanoparticles with both drug-binding and receptor-binding capacity. In comparison to FSI nanoparticles alone, the bifunctional nanoparticles demonstrate superior tumor binding in vitro and enhanced tumor suppression in vivo.

Figure 2.

Physicochemical characterization of ELP diblock copolymer nanoparticles. (a) SDS-PAGE was used to confirm the identity and purity of all ELPs (Table 1). (b) The CMT for all the four ELP diblocks was determined by UV-vis spectrophotometry, and the relationship between CMT and concentration was plotted. All four ELPs assemble nanoparticles above the indicated lines. (c) DLS was used to characterize the hydrodynamic radii (R_h) of 25 μM samples from 18 to 37 $^{\circ}\text{C}$. Above the CMT, each ELP diblock assembles nanoparticles with $R_h \approx 25$ nm. Below the CMT, ELPs remain unassembled with $R_h \approx 5$ nm ($n = 3$, mean $\pm$ SD).

Figure 3.

ISR and FSI spatial co-localization into bifunctional nanoparticles. (a) To explore the coassembly of ISR and FSI, the two diblock copolymers were fluorescently labeled, mixed in a 1:1 ratio, and imaged above the CMT (30 °C) using confocal laser scanning microscopy. When merged, FSI–CF (green), and ISR–Rho (red) show a high degree of spatial co-localization (yellow). (b) ImageJ and JACoP analysis confirmed a Pearson’s coefficient (PC) of 0.976, indicating the strong co-localization of two nanoparticles. (c) Cryo-TEM was used to observe ISR nanoparticles alone (left) or the 50% ISR and 50% FSI bifunctional nanoparticles (right). Both samples have a similar distribution of particle shape and have particles sizes consistent with DLS (Table 1). ISR nanoparticles have an average diameter of 33.8 ± 3.4 nm ($n = 6$, mean $\pm$ SD), and 50% ISR and 50% FSI nanoparticles have an average diameter of 33.7 ± 3.7 nm ($n = 6$, mean $\pm$ SD). Scale bar length = 100 nm.

Figure 4.

Cyclic RGD peptide competition with ISR-Rho for integrin receptor binding. (a) Confocal microscopy was used to observe ISR-Rho (red) binding ($20\ \mu\text{M}$, 60 min, $37\ ^\circ\text{C}$) to MDA-MB-468 cells after a preblocking incubation (60 min, $37\ ^\circ\text{C}$) with phosphate-buffered saline (PBS) or an excess of a cyclic RGD peptide (c-RGDfK). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Panels i–iii show the binding of ISR-Rho on cells preincubated with PBS. Panels iv–vi show the binding of ISR-Rho to cells preincubated with $125\ \mu\text{M}$ c-RGDfK. (b) Quantitative analysis of confocal images using ImageJ shows a significant difference in the fluorescent intensity of ISR-Rho preincubated with PBS or c-RGDfK (paired *t* test; *, $p = 0.01$). The significant decrease in rhodamine fluorescence suggests that both ISR-Rho and the cyclic peptide compete for the same population of cell-surface integrins. Values represent mean $\pm$ SD ($n = 3$).

Figure 5.

Bifunctional ISR/FSI nanoparticle recognition and binding of integrin receptors similar to ISR nanoparticles. Confocal microscopy was used to study the binding of 200 μM rhodamine-labeled nanoparticles (red) with or without coassembly for 1 h at 37 $^{\circ}\text{C}$. (a) MDA-MB-468 cells were incubated with ISR-Rho, FSI-Rho, and FSI-Rho mixed with unlabeled ISR (50% ISR and 50% FSI). Panels i–iii represent integrin binding with ISR-Rho. Panels iv–vi represents integrin binding with FSI-Rho and panels vii–ix represents integrin binding with 50% ISR and 50% FSI. Nuclei were stained with DAPI (blue). (b) Normalized fluorescence ($n = 3$, mean $\pm$ SD) showed statistically significant differences between ISR-Rho and FSI-Rho, and between ISR-Rho and 50% ISR and 50% FSI (Tukey's post-hoc test; $\alpha = 0.05$; ***, $p < 0.0001$; ○○○, $p < 0.0001$) and between 50% ISR and 50% FSI and FSI-Rho (Tukey's post-hoc test; $\alpha = 0.05$; ●●, $p < 0.0001$). (c) To further characterize binding, flow cytometry was used to identify the percent of cells positive for FSI-Rho assembled with increasing proportions of unlabeled ISR. For 50% ISR and 50% FSI, more than 90% of cells were positive, which was equivalent to that observed for cells treated with a positive ISR-Rho control.

Figure 6.

Bifunctional nanoparticles retaining of Rapa similarly to FSI nanoparticles. (a) Rapa loading of coassembled ISR/FSI nanoparticles is lower compared to FSI alone due to the reduced number of FKBP domains, whereas ISR alone failed to demonstrate high Rapa loading ($n = 3$, mean $\pm$ SD). (b) The coassembled nanoparticles remained colloidally stable with and without Rapa loading as assessed by DLS over 24 h at 37 °C ($n = 3$, mean $\pm$ SD). (c) Rapa-loaded formulations were then dialyzed to assess drug retention under sink conditions. Bifunctional ISR/FSI nanoparticles retained drug similarly to FSI nanoparticles. Even though the ISR alone can solubilize low levels of Rapa, it was unable to retain drug under sink dialysis conditions ($n = 3$, mean $\pm$ SD).

Figure 7.

FSI-Rapa and ISR/FSI-Rapa potency in inhibiting MDA-MB-468 proliferation in vitro compared to free Rapa. (a) The inhibitory concentration corresponding to 50% suppression of proliferation, IC₅₀, for FSI-Rapa, ISR/FSI-Rapa, and free Rapa were determined by nonlinear regression as 0.21 ± 0.02 , 0.32 ± 0.16 , and 1.81 ± 0.96 nM, respectively ($n = 3-4$, mean $\pm$ SD). (b) Tukey's post-hoc analysis revealed statistically significant differences in IC₅₀ between free Rapa and FSI-Rapa and ISR/FSI-Rapa each ($\alpha = 0.05$; *, $p = 0.0004$; **, $p = 0.001$).

Figure 8.

Tumor regression study demonstration of greater antitumor efficacy for the bifunctional nanoparticle than FSI-Rapa or free Rapa in breast tumor xenografts. Athymic nude mice with orthotopic 50–100 mm³ MDA-MB-468 tumors were treated three times a week intravenously. (a) SI-Rapa, free Rapa, and FSI-Rapa were compared at 0.25 mg/kg. Simultaneously, FSI-Rapa was evaluated for dose escalation from 0.0075–0.075 mg/kg, which revealed intermediate tumor suppression at the 0.075 mg/kg dose. The comparisons between all of the untargeted formulations gave significantly different tumor volumes on the last day of treatment only between PBS and FSI-Rapa at 0.25 mg/kg (Tukey's post-hoc analysis; $\alpha = 0.05$; ***, $p = 0.001$). (b) Based on the prior study, a comparative study was performed between the bifunctional and the untargeted formulations at an intermediate Rapa dose of 0.075 mg/kg. The bifunctional nanoparticle gave significant differences in tumor volume on the last day of treatment compared to PBS or free Rapa (Tukey's post-hoc analysis; $\alpha = 0.05$; **, $p = 0.003$; *, $p = 0.02$, respectively). (c) Analysis of this study using nonparametric survival analysis also demonstrated a significant extension of survival for the bifunctional ISR/FSI-Rapa treatment compared with PBS, free Rapa, and FSI-Rapa (log-rank post-hoc analysis; $\alpha = 0.008$; $p = 0.001$, 0.001, and 0.002, respectively). FSI-Rapa treatment also significantly prolonged survival compared to PBS ($\alpha = 0.008$, $p = 0.005$).

Figure 9.

Intratumoral accumulation of bifunctional ISR/FSI nanoparticles compared with controls. Athymic nude mice with orthotopic 50–150 mm³ MDA-MB-468 tumors were injected intravenously with 0.15 mg/kg rhodamine tagged FSI or ISR/FSI nanoparticles, and tumor accumulation was studied by quantifying fluorescence in tumor tissue sections excised at 2 and 8 h ($n = 3$ mice per group). (a) Tumor tissue sections at 2 h showed no rhodamine (red) fluorescence above PBS background. Distinct fluorescence was observed only for the bifunctional nanoparticle at 8 h relative to PBS, as indicated by white arrows. Nuclei were stained with DAPI (blue). A representative image is shown from each group. Scale bar = 20 μ m. (b) The quantified rhodamine fluorescence ($n = 6–12$, mean $\pm$ 95% CI) showed no significant difference above PBS background at 2 h. In contrast, the rhodamine fluorescence of ISR/FSI nanoparticles at 8 h was significantly greater compared with PBS, FSI at 2 h, and ISR/FSI at 2 h each (Tukey's post-hoc analysis; $\alpha = 0.05$; *, $p = 0.013$; **, $p = 0.004$; and $\circ\circ$, $p = 0.001$).

Table 1

Physicochemical Characterization of ELP Diblock Copolymers Evaluated in This Manuscript

label	amino acid sequence ^d	MW (kDa) ^b	purity (%) ^c	CMT (°C) ^d	R_h^e	
					20 °C (nm)	37 °C (nm)
SI	G(VPGSG) ₄₈ (VPGIG) ₄₈ Y	39.6	94.7	27.4	5.1 ± 0.8	23.3 ± 0.4
FSI	FKBP -G(VPGSG) ₄₈ (VPGIG) ₄₈ Y	51.4	99.1	25.0	4.8 ± 0.1	23.7 ± 0.1
IS	G(VPGIG) ₄₈ (VPGSG) ₄₈ Y	39.6	93.6	28.7	4.4 ± 0.5	23.9 ± 0.4
ISR	G(VPGIG) ₄₈ (VPGSG) ₄₈ Y- GRGDGG	40.1	93.8	26.5	4.3 ± 0.1	24.2 ± 0.3

^a**FKBP** amino acid sequence: GVQVETISPGDGRTPKRGQTCVVHYTGMLEDGKKFDSRRNKPFFKMLGKQEVIRGWEEGVAQMSVGQRAKLITISPDYAYGATGHPGHPHATLVFDVELLKE.

^b Estimated from open reading frame excluding the methionine start codon.

^c Purity was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis and densitometry analysis of the copper chloride stained gel using ImageJ.

^d CMT is the temperature above which 25 μM ELP in phosphate-buffered saline (PBS) assemble nanoparticles.

^e Hydrodynamic radii, R_h , measured by dynamic light scattering at 25 μM ELP concentration ($n = 3$, mean ± SD).

Pharmacokinetic Parameters and Tumor Bio-Distribution of FSI and of ISR/FSI in Nude Mice^a

Table 2

treatment	k (h ⁻¹)	$t_{1/2}$ (h)	C_0 (% ID/g)	V_d (mL)	CL (mL/h)	$C_{\text{tumor},8\text{h}}$ (% ID/g) ^b	n
FSI	0.8 ± 0.3	1.0 ± 0.3	19.0 ± 7.7	6.7 ± 4.4	5.3 ± 3.5	2.4 ± 1.8	11
ISR/FSI	0.7 ± 0.1	1.1 ± 0.2	11.1 ± 6.5	12.5 ± 7.4	8.2 ± 4.4	4.9 ± 3.1	8

^a microPET images were analyzed to estimate biodistribution and pharmacokinetic parameters for ⁶⁴Cu-labeled nanoparticles (Figure S3).

Pharmacokinetic parameters were estimated by fitting a single exponential decay to signal from 5 min to 2 h in individual mice, in which a log-linear decrease in the heart concentration (% ID/g) was clearly observed and well above background (Figure S4a). Between the two groups, there were no significant differences in the apparent elimination rate constant, k , the blood half-life, $t_{1/2}$, or the blood clearance, CL. Statistically significant differences were detected between treatments in the initial concentration, C_0 , and volume of distribution, V_d (t -test, $\alpha = 0.05$, and $p = 0.03$ and 0.05, respectively). Values indicate mean ± SD ($n = 8-11$).

^b After 8 h, when the blood was cleared of detectable signal, the final tumor accumulation was compared between treatments, $C_{\text{tumor},8\text{h}}$, which revealed that there was significantly more signal in ISR/FSI than in the FSI treatment group (t -test, $\alpha = 0.05$, and $p = 0.04$).