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Tripeptide-Stabilized Oil-in-Water Nanoemulsion of an Oleic Acids-Platinum(II) Conjugate as an Anticancer Nanomedicine

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Abstract

We report a nanoemulsion (NE) which is stabilized by self-assembling tripeptide lysine-tyrosine-phenylalanine (KYF) and encapsulates an oleic acids-platinum conjugate formed using simple Pt (II) coordination chemistry. The KYF-Pt-NE is evaluated both in cultured ovarian cancer cells and in an in vivo preclinical cancer model and shows pH dependent Pt (II) release, which is low at physiological pH and enhanced at tumoral pH. The biological activity of KYFPt-NE, evaluated in multiple ovarian cancer cell lines, is significantly higher when compared to the analogous Pt (II) complex used in the clinic. Concurrently, the KYF-Pt-NE platform shows good compatibility with the immune system. Preliminary in vivo testing of KYF-Pt-NE with tumor bearing mice indicates

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Supporting Information

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Detailed synthesis of oleic acids–Pt (II) conjugate and the KYF-Pt-NE, in vitro and in vivo methods, HRMS, ¹H NMR, ¹³C NMR, IR spectra, HPLC analysis, in vivo NIRF images (PDF)

Notes

The authors declare no competing financial interest.

efficient Pt (II) delivery to the tumor. Together, these results demonstrate the potential of peptide-stabilized nanoemulsions, specifically KYF-Pt-NE as an effective nanomedicine against cancer.

Graphical Abstract

Platinum-based drugs have been used in pre- or post-surgical adjuvant therapy or as the main treatment option for many malignancies.¹ Epithelial ovarian cancer is an important cause of cancer death in women and the survival rates have not changed dramatically over the past four decades. The standard of care encompasses surgical removal of the tumor followed by administration of Pt (II)-based agents.² The Pt (II) complexes target nuclear DNA and bind to nucleophilic N7-sites of nucleobases guanine and adenine, which interferes with DNA replication and transcription and leads to cellular apoptosis.³ Unfortunately, Pt (II) therapy is associated with high systemic toxicity. In addition, intracellular thiol containing molecules bind to Pt (II) metal, which results in deactivating about 60% of drug molecules, leaving only a small fraction able to enter the nucleus and thus available to therapeutically target nuclear DNA. This Pt (II) complex deactivation may result in increased DNA repair and development of resistance and, ultimately, treatment failure.⁴

Nanotechnology can address a number of current chemotherapy-related challenges as nanoparticles can solubilize or shield hydrophobic or highly toxic agents.⁵ Liposomal formulations of cisplatin⁶ as well as polymer-based platinum formulations, e.g., poly(lactic-co-glycolic acid) (PLGA),⁷ poly-(glutamic acid),⁸ or poly(lactic acid) (PLA),⁹ have been developed. Also, protein¹⁰ or inorganic carriers¹¹ have been reported. However, a potential limitation to future success is that the nanoparticle's building blocks are based on lipids or polymers that differ from each other in physicochemical properties and may lack biocompatibility and biodegradability, affecting the system's efficacy. Also, platforms such as protein conjugates have limited stability and their properties are not easily modified.¹²

The incorporation of biologically derived materials as nanoparticle building blocks should improve biocompatibility and biodegradability features. Short peptides are highly specific and versatile, have low toxicity profiles, and are safe, which prompted their clinical applications.¹³ In part, pharmaceutical interest in peptides is based on their small size, sequence tunability, and large-scale synthesis at low cost, which makes them comparable to

small molecule drugs.¹⁴ Presently, peptide-based therapies are being exploited for use in highest mortality diseases including cancer,¹⁵ cardiovascular diseases,¹⁶ and also for certain metabolic disorders¹⁷ and infections.¹⁸ Most of the above approaches are focused on biological activity of peptides, i.e., their use as therapeutic agents. There are additional opportunities in the use of short self-assembling peptides as structural components for a variety of biomedical applications.^{18–22}

It is increasingly appreciated that even short peptides (two or three amino acids) contain sufficient chemical information to form supramolecular structures, including gels and emulsions, with rich sequence-dependent properties.^{23–25} For example, the self-assembling ability of unprotected tripeptides, such as lysine-tyrosine-phenylalanine (KYF), can be utilized at an oil/water interface to create stabilized nanofibrous networks with properties that are dictated by the peptide sequence.²⁶

Here, we report a tripeptide lysine-tyrosine-phenylalanine (KYF) stabilized nanoemulsion with Pt (II) therapy (KYF-Pt-NE), and the core is composed of oleic acids–Pt (II) conjugate. Importantly, this is the first demonstration, to the best of our knowledge, of peptide stabilization of a nanoscale emulsion. A number of key features of this nanostructure provide unique properties, which are expected to reduce barriers to its clinical translation. First, oleic acid is a pharmaceutical excipient due to its nontoxicity and biocompatibility.²⁷ Second, both the KYF and oleic acid are biodegradable, and finally, the amino acid breakdown products of the tripeptide and oleic acid have FDA Generally Recognized As Safe (GRAS) status, which reduces future barriers to their clinical applications.

RESULTS AND DISCUSSION

The KYF-Pt-NE is presented in Figure 1. We formed an oleic acids–(COOH)₂Pt(II)(NH₃)₂ conjugate (top), wherein carboxylate groups serve as coordinating ligands to the Pt (II) center to resemble clinically relevant carboplatin. This is expected to preserve the Pt (II) mechanism of action and formation of active cis-[Pt(NH₃)₂(H₂O)₂]²⁺ moiety that enters the nucleus.² The conjugate's purity, determined by NMR (¹³C and ¹H), was 99%. The ¹⁹⁵Pt NMR (Figure S7) shows a single peak at –2824 ppm confirming the presence of bisubstituted oleic acids–Pt (II) conjugate. The oleic acids–Pt (II) conjugate was also characterized with HRMS and the spectrum is presented in Figure S4. The exact mass of the conjugate is 791.5140 au, which was identified in the spectrum and together with isotopic distribution confirms the molecular formula. Additionally, the elemental analysis of oleic acids–Pt (II) conjugate (Supporting Figure S9) for C, H, N shows good agreement between the theoretical and experimentally found values providing additional confirmation of the purity of oleic acids–Pt (II) conjugate. The KYF-Pt-NE was formed directly from the conjugate in the presence of KYF tripeptide, via a nanoprecipitation method.²⁸ Shortly, the oleic acids–Pt (II) conjugate in isopropanol was added dropwise to an aqueous solution of KYF tripeptide, at 1:3 molar ratio of KYF to oleic acids–Pt (II) conjugate, with moderate stirring. The KYF tripeptide is soluble in water at 37 °C and at neutral pH of 7; thus, the synthesis of KYF-Pt-NE was accomplished at physiological conditions.

The physicochemical parameters of KYF-Pt-NE were characterized using a number of complementary techniques. The size of KYF-Pt-NE and morphology were determined by dynamic light spectroscopy (DLS) and transmission electron microscopy (TEM). The average hydrodynamic diameter of the KYF-Pt-NE measured by DLS is 240 nm with a low polydispersity of 0.156. The TEM image of KYF-Pt-NE is presented in Figure 1 (bottom right) and shows that KYF-Pt-NE have a spherical morphology and their core diameter is 107 ± 27 nm. This size range of KYF-Pt-NE is suitable for in vivo applications,²⁹ as small-sized nanoparticles extravasate through leaky tumor vasculature into the tumor's interstitium: a phenomenon known as enhanced permeability and retention (EPR) effect or passive targeting.³⁰ The zeta potential of KYFPt-NE is -60.1 mV, indicating good colloidal stability of KYFPt-NE. The negative surface charge can be attributed to the oleic acid COO^- groups at the KYF-Pt-NE's surface, as the KYF peptide is positively charged at pH 7. A negative surface charge is desirable since it would be predicted to prevent nonspecific electrostatic interactions with proteins in the blood compartment that might lead to cytotoxicity.²⁹

We next examined if the KYF peptide is indeed necessary to form and stabilize the nanoemulsion. We used the oleic acids–Pt (II) conjugate only, without the presence of KYF peptide during the synthesis. We found that the Pt-NE forms with an average size of 790 nm, measured by DLS, and polydispersity of 0.239. The TEM image of Pt-NE is shown in Figure S10. The zeta potential of Pt-NE is -47.09 mV. This is ~ 13 mV less than that of KYF-Pt-NE. Figure 2A shows the solutions of Pt-NE and KYF-Pt-NE. It can be seen that the KYF coassembly rendered a cloudy appearance. However, the Pt-NE aggregates just after a few hours (Figure 2B) while the KYF-Pt-NE remains stable. Moreover, the diameter of KYF-Pt-NE does not significantly change even after several months of storage (Figure S11). This suggests that KYF peptide is essential for stabilizing the KYF-Pt-NE. We propose that the enhanced stabilization of the KYF nanoemulsion's surface relates to formation of a nanofibrous network, similar to previously reported tripeptide-stabilized emulsions;²⁶ comprehensive modeling studies are currently underway to assess these interactions.

The Pt (II) concentration in KYF-Pt-NE was analyzed using atomic absorption spectroscopy (AAS) and was found to be 10 wt %. We performed preliminary drug release studies with KYF-Pt-NE at three specific pH values: 7.4, 6.8, and 5.0. These pH values are physiologically relevant: physiologic pH is 7.4, while the pH values of tumor interstitium and cancer endosomes are 6.8³¹ and 5.0,³² respectively. The acid hydrolysis of carboplatin is well studied and involves successive displacement of two monodentate carboxylates, starting with a ring-opening step, followed by a complete loss of ligand.^{33,34} The acidity of the aqueous medium influences the rate of both steps, and at low acidity the first step is ten times faster than the second one, while at high acidity this difference is 4-fold.³⁵ If an acid or other nucleophile is not present in the media, the complex is inert and the displacement of carboxylate ligands does not occur.³⁴ The results of the drug release studies are presented in Figure 2C. During the first 4 h, 20.8% of Pt (II) was released from KYF-Pt-NE at pH 7.4, 32.8% at pH 6.8, and 47.5% at pH 5.0. After 24 h, 44.4% and 46.9% of Pt (II) was released at pH 7.4 and 6.8, respectively, and 64.7% of Pt (II) at pH 5.0. At the final time point (6 days), the KYF-Pt-NE released 72.8% of Pt (II) at pH 7.4, 78.0% of Pt (II) at pH 6.8, and

82.5% of Pt (II) at pH 5.0. The highest difference in Pt (II) release, 12% between pH 7.4 and 6.8 as well as 26.7% between pH 7.4 and 5.0, is observed during the first 4 h. This corresponds to the crucial time after the intravenous drug administration, of the toxicity buildup in the system.

These results demonstrate that Pt (II) release is significantly pH-dependent. Release is slowest at physiological pH. Thus, effective Pt (II) shielding can be achieved in the circulation at pH 7.4 and accelerated Pt (II) release can be expected in the tumor microenvironment and within the cancer cell.

To confirm the capability of KYF-Pt-NE for intracellular drug delivery, we conjugated the FITC fluorophore to the N terminus of KYF at the end of solid-state peptide synthesis. To synthesize FITC labeled KYF-Pt-NE, 50 mol % of the total peptide content was replaced with FITC-KYF. The fluorescently labeled nanoemulsions were stable for storage for several months (Figure S16). The FITC-KYF-Pt-NE were tested using a number of ovarian cancer-derived cell lines. We specifically used the isogenic ovarian cancer cell lines A2780 (Pt sensitive) and CP70 (Pt resistant)^{36–38} as well as ovarian cell lines with diverse genomic features, histotypes, and varying degrees of Pt (II) resistance.^{39–41} All cells were incubated with FITC-KYF-Pt-NE for 15 min, and the corresponding confocal images are shown in Figure 3. The FITC-KYF-Pt-NEs (green) quickly enter cells via endocytic-based mechanisms and the nanoemulsions can be seen distributed throughout the cytosol. At this early time point, the FITC-KYF-Pt-NEs were not yet found to be associated with lysosomes (red). Importantly, the uptake of FITC-KYF-Pt-NEs by all cell lines suggests that the nanoemulsions can be used as a drug delivery system.

We next examined the biological activity of KYF-Pt-NE using the same 6 cell lines. To this end, the cells were incubated with KYF-Pt-NE for 72 h and the cellular viability was determined after the incubation using an MTT assay. The results were compared to controls, which included carboplatin, KYF-NE without Pt (II), cells only, cisplatin, and oleic acids–Pt (II) conjugate. The concentrations used correspond to IC₅₀ values of KYF-Pt-NE obtained independently for each cell line (Figure S19; the IC₅₀ values for carboplatin are shown for comparison in Figure S20). The samples were run in triplicate and the experiment was performed three times. The results of biological activity of KYF-Pt-NE are shown in Figure 4. The viability of Pt (II) sensitive A2780 and Pt (II) resistant CP70 cells was reduced by 44.3% and 46.2%, respectively, after incubation with KYF-Pt-NE. Carboplatin, the clinically relevant analogue, decreased the viability of A2780 cells by 18.5% and CP70 cells by 9.6% only. The viability of Pt (II) sensitive TOV-21G cells was reduced by 55.9% after incubation with KYF-Pt-NE, while carboplatin resulted in lowering the viability by just 16.5%. In OV-90 cells, characterized with intermediate Pt (II) resistance, the KYF-Pt-NE decreased the viability by 55.3% and carboplatin by just 23.9%. The viability of two resistant cancer cell lines ES-2 and SKOV-3 was reduced by 45.9% and 54.3%, respectively, after incubation with KYF-Pt-NE and carboplatin reduced the viability by 10.3% in ES2 cells and by 16.8% in SKOV-3. In all tests, and suggestive of its therapeutic candidacy, the KYF-Pt-NE had a greater effect on cell death than just the use of carboplatin. Overall, the efficacy of KYF-Pt-NE was higher by 25.8% in A2780 cells, 36.6% in CP70, 31.4% in OV-90, and 35.6% and 37.5% in ES-2 and SKOV-3 cells, respectively. The greatest therapeutic enhancement of

39.4% was observed in TOV-21G cells. In addition, we compared the biological activity of KYF-Pt-NE to cisplatin, a Pt (II)-based agent which is no longer favored in the clinic because of its systemic toxicity profile.⁴² In our studies, KYF-Pt-NE affected viability significantly greater than cisplatin in two cell lines, SKOV-3 and TOV-21G, by approximately 15% and 40%, respectively. The activities of both KYF-Pt-NE and cisplatin were comparable in A2780, CP70, and OV-90 cells, and cisplatin activity was higher by 35% in the ES-2 cell line when compared to KYF-Pt-NE. The oleic acids–Pt (II) conjugate was also found to be biologically active, resulting in comparable cytotoxicity to KYF-Pt-NE in the isogenic cell lines A2780 and CP70. In the remaining cells, however, higher reduction in viability was observed for KYF-Pt-NE than for oleic acids–Pt (II) conjugate, by ~20% in TOV-21G, 45% in OV-90, 30% in ES-2, and 15% in SKOV-3 cells, highlighting the importance of the particulate formulation in inducing greater therapeutic effects.

We also evaluated if KYF-Pt-NE induces the nitric oxide production by macrophages following Method ITA-7 defined by the NIH's Nanotechnology Characterization Laboratory. Nitric oxide can interact with various molecular targets in vivo resulting in cytotoxicity.⁴³ We performed the detection of nitric oxide secretion by macrophage cell line RAW 264.7 in response to KYF-Pt-NE. We used different concentrations of KYF-Pt-NE with respect to the theoretical Pt (II) human plasma concentration (0.021 mg/mL) during therapy. The results were compared to controls and data are presented in Figure S12. In all tests, nitric oxide levels produced due to KYF-Pt-NE were comparable to negative control.

Finally, and as a prelude to therapeutic testing, we assessed the suitability of KYF-Pt-NE for biological applications using an in vivo, preclinical ovarian cancer mouse model. For these experiments, we first determined the serum stability of KYF-Pt-NE (Figure 5A). We detected no evidence of KYF-Pt-NE opsonisation in 20% serum after 1 day of incubation.

We then determined the in vivo half-life of KYF-Pt-NE. We conjugated the Cy7 near-infrared fluorophore to KYF, and 5 mol % of Cy7-KYF, with respect to total tripeptide content, was used to synthesize the Cy7-KYF-Pt-NE. We found that the presence of Cy7-KYF in the nanoemulsion's coating did not influence the stability of the construct (Figure S18). The Cy7-KYF-Pt-NE were administered intravenously at Pt (II) concentration of 0.0162 mg/g. This dose corresponds to Pt (II) concentration in FDA dose of cisplatin (75 mg/m²) for patients, converted to its equivalent dose for mice according to Reagan-Shaw et al.⁴⁴ Blood samples (20 μ L) were collected at 5, 15, 30, and 90 min post injection, and the Cy7-KYF-Pt-NE circulation was assessed by two methods: fluorescence and AAS. Based upon this analysis, we calculated the in vivo half-life of Cy7-KYF-Pt-NE to be 30 min.

We next examined the in vivo biodistribution of Cy7-KYFPt-NE in ovarian tumor-bearing mice. The Cy7-KYF-Pt-NE were injected intravenously via tail vein at the same concentration of Pt (II), as in the half-life test. The animals were imaged with near-infrared fluorescence (NIRF) 24 h post-injection. We did not observe the visual NIRF signal from Cy7-KYF-Pt-NE in the tumor while imaging the animals (Figure S21). However, the excised tumors were assessed with NIRF regions of interest (ROI) and compared to the control tumor from a non-injected mouse. The NIRF values were higher for tumors obtained from mice injected with Cy7-KYFPt-NE versus the control, and the representative image is shown

in Figure 5B. In addition, the complementary AAS analysis of the same tumors revealed the presence of 0.284 μg of platinum in the tumor from injected mouse and negligible Pt (II) concentration in the control tumor. These data suggest that Cy7-KYF-Pt-NE extravasates into the tumor interstitium to deliver Pt (II) therapy, but the low NIRF signal might be indicative of the enzymatic degradation of KYF and renal clearance. We also performed a comprehensive evaluation of Cy7-KYF-Pt-NE distribution into vital organs. The NIRF image of the organs is presented in Figure S22A. The Cy7-KYF-Pt-NE were found in the liver, spleen, and lungs. The quantitative AAS measurements (Figure S22B) revealed that Pt (II) also accumulated in a lesser amount in the kidneys and in the heart. The Pt (II) was not detected in the brain, which indicates that the Cy7-KYF-Pt-NE did not cross the blood-brain-barrier.

CONCLUSION

In conclusion, we herein present a physicochemical and biological evaluation of a newly developed KYF-Pt-NE platform that integrates self-assembled tripeptides as emulsion-stabilizing structural components. The physicochemical parameters and efficacy of KYF-Pt-NE are suitable for drug delivery vehicles and in vivo studies show Pt (II) delivery to the tumor tissue. Importantly, the platform can be easily extended to incorporate other functional peptides into the nanostructure. Ongoing studies will investigate the peptide-nanoemulsion stabilization phenomenon as well as the in vivo fate of KYF-Pt-NE and its therapeutic potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Oleic acids–Pt (II) conjugate (top). Schematic representation of KYF tripeptide-platinum(II) nanoemulsion (KYFPt-NE) (bottom). TEM image of KYF-Pt-NE (bottom right).

Figure 2.

(A) Picture of Pt-NE and KYF-Pt-NE in water. (B) Stability tests for Pt-NE and KYF-Pt-NE in water. (C) Pt (II) release from KYF-Pt-NE in vitro at different pH (in PBS).

Figure 3.
Confocal images of cancer cells incubated with FITC-KYF-Pt-NEs (green); nuclei are stained with DAPI and lysosomes are red. Scale bars are 10 μm .

Figure 4.

Cellular viability of different ovarian cancer cell lines after 72 h incubation with KYF-Pt-NE. Each column represents the mean and standard deviation of $N=3$ and $p < 0.005$. The concentrations are constant in each cell line, and are as follows: $4.92 \mu\text{M}$ (A2780), $8.80 \mu\text{M}$ (CP70), $2.46 \mu\text{M}$ (TOV-21G), $9.84 \mu\text{M}$ (SKOV3), $7.38 \mu\text{M}$ (ES-2), and $19.7 \mu\text{M}$ (OV-90). The concentration of KYF-Pt-NE and oleic acids–Pt (II) conjugate was adjusted with respect to Pt (II) content measured by Pt AAS. Abbreviations: Carbpt — carboplatin; KYF-NE — KYF tripeptide-coated nanoemulsion; KYF-Pt-NE — KYF tripeptide-coated nanoemulsion containing Pt (II); Cispt — cisplatin; Pt-oleic — oleic acids–Pt (II) conjugate.

Figure 5.

(A) Stability of KYF-Pt-NE in 20% serum. (B) Tumors excised from a mouse injected with Cy7-KYF-Pt-NE (left) and control (right). The data were collected with the help of the Small Animal Imaging Center in the Translational and Molecular Imaging Institute.