pH-Activated Size Reduction of Large Compound Nanoparticles for *in vivo* Nucleus-Targeted Drug Delivery

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Abstract Nucleus-targeted drug delivery is a promising strategy for anticancer therapy, but *in vivo* nucleus-targeted drug delivery has been challenging. Limited by the channel size of the nucleopore, vehicles that enter the nucleus *via* the nucleopore actively should be small and decorated with nuclear localization signal (NLS). However, the small vehicle size may promote leakage of vehicles into normal tissues, and the positively-charged NLS can lead to strong non-specific interactions *in vivo*. In the present study, we demonstrate an *in vivo* nucleus-targeted drug delivery using large compound nanoparticles with detachable PEG shell. The nanoparticles are composed of PEG-benzoic imine-oligo-*L*-lysine/iridium(III) metallodrug complex and formed in a kinetically-controlled fashion. Under physiological conditions (pH 7.4), the nanoparticles are large (ca. 150 nm) and protected by an inert PEG shell. When internalized into intracellular acidic endo/lysosomes of cancer cells, the nanoparticles dissociate into smaller ones (ca. 40 nm) and the PEG chains detach due to the cleavage of the benzoic imine bond at low pH. The small nanoparticles, with exposure of the oligo-*L*-lysine after the detachment of the PEG shield, then translocate into the nucleus *via* the nucleopore due to the small size and nuclear localization ability of the oligo-*L*-lysine. Importantly, the small particles could significantly release the contained drug into the nucleus, leading to ca. 20-fold higher cytotoxicity compared to the native drug *in vitro*. Further *in vivo* application of the nucleus-targeting nano-system in a nude-mice model showed significant tumor inhibition and remarkable life-span elongation.

Key Words iridium (III), nanomedicine, cancer therapy, nucleus-targeting

Introduction

Nanocarriers incorporating anticancer agents can potentially mitigate the side effects of conventional chemotherapy by selectively delivering these agents to regions of disease [1] and minimizing the dosage required for efficacy [2]. Typically, the vehicles release molecules into the cytosol [3], and not into the nucleus, where most anticancer drugs exert their effects [4]. Although the drugs released into the cytosol can enter the nucleus by simple diffusion [5], cancer cells, especially drug-resistant cancer cells, can evolve various intracellular drug-resistance mechanisms to limit the nuclear entry of cytosolic drugs [4-7]. Consequently, only a small percentage of drugs

delivered into the cytosol finally reach the nucleus [4,8]. Thus, designing and preparing drug-containing nanocarriers that are capable of translocating into the nucleus of cancer cells and then releasing the drug inside the nucleus could dramatically improve the efficacy of therapy [3,4,7,9,10].

Usually, cargoes enter the nucleus either when the cell undergoes mitosis (during mitosis, the nuclear membrane, the only barrier between the cytosol and nucleus, temporarily disassembles) or *via* the nucleopore [3]. Nuclear entry of cargoes *via* the nucleopore pathway is more efficient as the cargoes can take advantage of both the mitosis and nucleopore pathways for nuclear entry. Limited by the channel size of the nucleopore, cargoes larger than the channel size are prevented from crossing the nuclear envelope *via* the nucleopore [11], while those smaller than the channel size but larger than 10 nm can go through the nucleopore only when they bear a nuclear localization signal (NLS) on the surface [7,9,10]. Although cargoes smaller than 10 nm can diffuse freely through the nucleopore without a NLS, the nuclear entry will be greatly promoted in the presence of a NLS [10,11]. Therefore, cargoes capable of going through the nucleopore efficiently should be small (less than 50 nm [9a,12]) and have a NLS on the surface. For example, it has been reported that the conjugates of anticancer drugs and a NLS using intranuclear-cleavable conjugation bonds can go through the nucleopore efficiently and reach the nucleus of cancer cells, and then release the drug there [7]. However, almost all of the drug conjugates that have been reported to enter the nucleus are small (*ca.* few nanometers), resulting in susceptibility to excretion by the kidneys [13] and unintentional leakage into other sites (*e. g.* capillaries and normal tissues) [14]. Furthermore, cleavage of the conjugation bonds in the lysosome rather than in the nucleus further decreases the efficiency of nuclear delivery.

Design of nanosystems for *in vivo* nucleus-targeted drug delivery has been challenging due to incompatible physiochemical requirements on the nanosystems going through in sequence of blood, extracellular matrix, and cytoplasm to ultimately the intracellular nucleus [3]. Recently, NLS-decorated nanoparticles have been explored as nanosystems for *in vivo* nucleus-targeted drug/gene delivery. These systems include charge-reversible magnetic mesoporous silica nanoparticles with TAT peptide [12], membrane-core nanoparticles with arginine-rich peptide [15], and mesoporous silica nanoparticles with TAT and RGD peptides [16,17]. However, most of the nanoparticles are small in diameter (less than 50 nm) which may promote unintentional leakage from vasculature into normal tissues and cause systematic toxicity [14]. Besides, the NLS is usually positively charged and thus could elicit strong non-specific interactions *in vivo* [3,18]. In the present study, to meet with the requirements of nanoparticles for *in vivo* applications and nuclear entry *via* the nucleopore, we designed and prepared kinetically-controlled large compound

nanoparticles with detachable PEG shell and folate decoration (denoted as LNP_{dePEG-FA}). LNP_{dePEG-FA} results from self-assembly of PEG-benzoic imine-oligo-*L*-lysine/iridium(III) metallodrug complex and further folate-functionalization of the nanoparticles. LNP_{dePEG-FA} has a large size of *ca.* 150 nm and bears an inert PEG shell under neutral conditions. Interestingly, after internalization into the acidic endo/lysosomes of cancer cells, LNP_{dePEG-FA} dissociates into smaller nanoparticles (SNPs, *ca.* 40 nm) and meanwhile the PEG chains are detached due to breakage of the benzoic imine bond. The detachment of PEG chains makes the previously shielded NLS oligo-*L*-lysine exposed then. So SNPs with oligo-*L*-lysine on their surfaces can enter the nucleus *via* the nucleopore due to the small size and the nuclear localization ability of oligo-*L*-lysine. LNP_{dePEG-FA} leads to *ca.* 20-fold higher toxicity *in vitro*, greater tumor suppression in mice and a much longer life-span of the mice compared to the native drug, indicating the ability of the nucleus-targeting LNP_{dePEG-FA} for cancer therapy.

Scheme 1 (A) Schematic illustration of the preparation of the kinetically-controlled large compound nanoparticles (LNP_{dePEG-FA}). (B) The dynamic behavior of LNP_{dePEG-FA} passing through multiple barriers from vascular environment to the nucleus. (1) LNP_{dePEG-FA} binds to tumor cells due to the folate-folate receptor affinity, (2) becomes internalized by receptor-mediated endocytosis, (3) dissociates into smaller nanoparticles (SNPs) and detaches from PEG chains upon endo/lysosome acidification.

(4) The small nanoparticle SNP escapes into the cytosol, (5) translocates into the nucleus *via* the nucleopore with oligo-*L*-lysine acting as a NLS, and (6) releases Ir(III) metallodrug in the nucleus by DNA/SNP interaction and induces cell death.

Results and discussion

Design and synthesis of PEG-*b*-oligo-*L*-lysine/iridium(III) amphiphilic complexes

A potential anti-cancer drug iridium (III) complex was chosen in this study due to its high hydrophobicity that drives self-assembly of its amphiphilic complexes with the double-hydrophilic block copolymer in water, affinity to His residues of proteins [19], significant cytotoxicity [20] and good fluorescence property for ease of tracking [24]. Due to hydrophobicity of the native drug, we designed and synthesized an amphiphilic complex of the metallodrug iridium(III) with PEG-*b*-oligo-*L*-lysine double-hydrophilic block copolymer to enhance drug solubility and form a nucleus-targeting drug delivery system. The PEG-*b*-oligo-*L*-lysine copolymer (Lys₃₀-CH=N-PEG-NH₂) contains four functional moieties, terminal amine groups for conjugation with a cell penetrating moiety FA [21], oligo-*L*-lysine Lys₃₀ as a NLS [22], pH responsive [23] and the metallodrug reactive moiety [24], PEG to protect against non-specific interactions *in vivo*, and benzoic imine bonds as pH-cleavable linkers [25] between PEG and oligo-*L*-lysine blocks (Scheme 1a).

Scheme 2 Synthetic route of the detachable, amine-terminated, PEGylated block copolymer Lys₃₀-CH=N-PEG-NH₂.

The synthetic routine of the detachable, amine-terminated, PEGylated block copolymer Lys₃₀-CH=N-PEG-NH₂ (**6**) was shown in Scheme 2 (the details of all the synthesis are described in the supporting information (SI)). For synthesis of the block copolymer, amine-terminated oligo(trifluoroacetyl-*L*-lysine), Lys(TFA)₃₀-NH₂ (**2**, Scheme 2), with a degree of polymerization of 30 was firstly synthesized *via* ring-opening polymerization of trifluoroacetyl-*L*-lysine-*N*-carboxy-anhydride (**1**) using *n*-hexamine as the initiator (confirmed by ¹H NMR analysis; Fig. SNMR-1 in SI). The terminal amine of Lys(TFA)₃₀-NH₂ (**2**) was then converted into a hydroxyl group based on the amide formation between glycolic acid (OH-CH₂-CO₂H) and Lys(TFA)₃₀-NH₂ to give a hydroxyl-terminated Lys(TFA)₃₀-OH (**3**, Scheme

2); *ca.* 92% of conversion; Fig. SNMR-2 in SI). Then, Lys(*TFA*)₃₀-OH (**3**) was reacted with 4-carboxybenzaldehyde to convert Lys(*TFA*)₃₀-CHO (**4**, Scheme 2) with a terminal aldehyde group, based on the esterification reaction between the carboxylic group of carboxybenzaldehyde and hydroxyl group of Lys(*TFA*)₃₀-OH (**3**) (*ca.* 74% of conversion; Fig. SNMR-3 in SI). The final block copolymer Lys₃₀-CH=N-PEG-NH₂ (**6**) was then obtained after the imine bond formation between the benzoic aldehyde group of Lys(*TFA*)₃₀-CHO (**4**) and the NH₂ group of 9-fluorenylmethoxycarbonyl mono-protected diamine polyethylene glycol (*fmoc*-HN-PEG-NH₂), followed by de-protection of the *fmoc* and trifluoroacetyl groups and dialysis to remove unreacted PEG and Lys₃₀ (Scheme 2). ¹H NMR spectroscopy and gel permeation chromatography (GPC) were used to characterize the polymers and the results confirmed significant formation of the benzoic imine bond and the resultant PEG/oligo-*L*-lysine block copolymer, respectively (Fig. SNMR-4 and Fig. S1A in SI).

Lys₃₀-CH=N-PEG-NH₂/iridium(III) complex was then synthesized *via* a 12-h reaction of the intermediate [Ir(dfppy)₂μ-Cl]₂ (dfppy = 2,4-difluorophenyl-2-pyridine) and the block copolymer Lys₃₀-CH=N-PEG-NH₂ in a mixture solvent of 2-ethoxyethanol/H₂O (3:1, *v/v*) at 110 °C under argon atmosphere (Scheme S7 in SI) [24]. In the complex, the highly hydrophobic Ir(III) moieties were grafted to part of the Lys₃₀ block *via* N-Ir coordination bond formation (Scheme 1), thus imparting an amphiphilic nature to the complex.

Preparation and characterization of the kinetically controlled large compound nanoparticles

Kinetically controlled large compound nanoparticles were subsequently prepared by diluting the Lys₃₀-CH=N-PEG-NH₂/Ir(III) complex in the mixture solvent with neutral water (pH 7.4) to cause abrupt assembly of the complex. Large compound nanoparticles with a detachable PEG shell and folate decoration (LNP_{dePEG-FA}) were then obtained after reaction of the nanoparticles with *N*-hydroxysuccinimide ester of folate (FA-NHS) and dialysis of the particle suspension against water at pH 7.4 (Scheme 1A). As determined by ICP-MS, the payload and encapsulation efficiency of Ir(dfppy)₂⁺ in LNP_{dePEG-FA} were found to be 10.6% (w/w) and 61.0%, respectively.

Fig. 1 (A) ¹H NMR spectra of Lys₃₀-CH=N-PEG-NH₂ at pH 7.4 and 6.0 in D₂O. (B) DLS results of LNP_{dePEG-FA} at pH values of 7.4, 6.0 and 4.0 in water. TEM images of LNP_{dePEG-FA} at pH 7.4 (C) and 4.0 (D) in water.

¹H NMR experiments were performed to test the pH-responsiveness of the benzoic imine bond in PEG/Lys₃₀ block copolymer. Fig. 1A shows that when the pH value was changed to 6.0, the signal at 7.8 ppm and pH 7.4 corresponding to the benzoic imine bond weakened by 64% accompanied by the appearance of a sharp signal at 10.1 ppm corresponding to the benzoic anhydride. This indicated significant rupture of the benzoic imine bond at a relatively low pH [25]. To test whether the benzoic imine bond in LNP_{dePEG-FA} can be cleaved intracellularly, large compound nanoparticles with non-detachable PEG chains (LNP_{PEG-FA}), as a control, were prepared under the same conditions and through the same processes as those for LNP_{dePEG-FA} except that NH₂-PEG-Lys₃₈ (Scheme S6 in SI) was used in place of Lys₃₀-CH=N-PEG-NH₂. The PEG chains of both LNP_{dePEG-FA} and LNP_{PEG-FA} were further tagged with the fluorophore Rhodamine B isothiocyanate (RBITC; see Section 7 in SI for experimental details). For either LNP_{dePEG-FA} or LNP_{PEG-FA}, confocal fluorescent microscopy revealed that the luminescence of RBITC (orange) from PEG was highly correlated with the luminescence from Ir(dfppy)₂⁺ (cyan) after 0.5 h incubation in HeLa cells (Fig. S3 in SI). After prolonging the incubation time to 2 h, a significant phase separation between PEG and Ir(dfppy)₂⁺ was observed for LNP_{dePEG-FA} (Fig. 2A), whereas the luminescence of RBITC from PEG still highly overlapped with that from Ir(dfppy)₂⁺ for LNP_{PEG-FA} (Fig. 2B). These results indicate the rupture of benzoic imine bond and detachment of PEG chains for LNP_{dePEG-FA} in the intracellular environment.

The molar ratios of Ir(dfppy)₂⁺ to amino groups in LNP_{dePEG-FA} and LNP_{PEG-FA} were determined to be 4.6 : 30 and 5.8 : 38 by ICP-MS, leaving *ca.* 70% unreacted -NH₂ groups (Table S1 in SI). TEM observation revealed that the LNP_{dePEG-FA} and LNP_{PEG-FA} formed in water at pH 7.4 were

spherical particles sized approximately 150 and 175 nm, respectively (Fig. 1C and Fig. S2B in SI). The average hydrodynamic diameters of LNP_{dePEG-FA} and LNP_{PEG-FA} in water at pH 7.4 was measured by DLS to be 170 nm and 196 nm, respectively, which are close to the results of the TEM observations (Fig. 1B and Fig. S2A in SI). However, when the pH gradually decreased to 4.0, the average hydrodynamic diameter of LNP_{dePEG-FA} and LNP_{PEG-FA} decreased significantly (Fig. 1B and Fig. S2A in SI) and the sizes became *ca.* 40 and 45 nm, respectively (Fig. 1D and Fig. S2C in SI). Previous studies on poly-*L*-Lys based nanoparticles reported similar behaviors of size reduction at low pH.²³ The size reduction at low pH indicates the dissociation of the primary large nanoparticles. TEM observations reveal that LNP_{dePEG-FA} (or LNP_{PEG-FA}) at pH 7.4 was a large assembly of a number of super-small particles (*ca.* 2~5 nm in diameter, the insets in Fig. 1C and Fig. S2B in SI). However, when the pH value decreased to 4.0, smaller particle-assemblies composed of much fewer super-small particles were observed (the insets in Fig. 1D and Fig. S2C in SI). To test whether this pH-triggered dissociation could occur intracellularly, TEM images of ultrathin sections of HeLa cells incubated with LNP_{dePEG-FA} for 0.5 and 2 h were recorded. The endocytosis of large nanoparticles by cancer cells at 0.5 h and formation of the small nanoparticles (SNPs, representing the small nanoparticles produced from the dissociation of LNP_{dePEG-FA}) as well as their escape from endo/lysosome at 2 h were observed (Fig. 2C and 2D). To understand the mechanism for the formation and dissociation of the large particles, zeta potential measurements were performed at pH 7.4~4.0. The results revealed that both LNP_{dePEG-FA} and LNP_{PEG-FA} became more and more positively charged with decreasing the pH values, and the positive charge became significant at pH 5.0~4.0 (Table S2 in SI). This suggests that the deprotonation of the amino groups in LNP_{dePEG-FA} (or LNP_{PEG-FA}) was facilitated at high pH [26] and the protonation degree became significantly higher at low pH [27]. The higher degree of protonation at low pH makes the oligo-*L*-Lys segments more hydrophilic and more electrostatically repelling to each other, leading to the dissociation of the large particle assemblies into the small particle assemblies. More importantly, when SNP (or SNP_{PEG-FA}, representing the small nanoparticles produced from the dissociation of LNP_{PEG-FA}) escaped from the endo/lysosomes (pH 6.0~4.0) into the cytosol (pH ~7.4), the nanoparticles were nearly non-charged but their size remained small (Fig. 2D). This suggests that the large nanoparticles (LNP_{dePEG-FA} and LNP_{PEG-FA}) were large compound nanoparticles formed in a kinetically-controlled fashion due to the abrupt adding of neutral water and fast assembly of the particles [28,29]. The kinetically controlled process leads to encapsulation of hydrophilic Lys moieties, resulting in the large size. At low pH, the amino groups from oligo-*L*-Lys segments in SNP that were kinetically encapsulated within the primary large compound nanoparticles are now

exposed to water and become protonated to stabilize the small nanoparticles. Although the pH value was changed back to ~ 7.4 , the hydrophilic nature of the amino groups helped to prevent the small nanoparticles from re-aggregating into large nanoparticles.

Fig. 2 Confocal luminescence images of HeLa cells incubated with (A) LNP_{dePEG-FA} or (B) LNP_{PEG-FA} (Ir: 2.0 mg/L) for 2.0 h at 37 °C (for RBITC: $\lambda_{\text{ex}} = 543$ nm, $\lambda_{\text{em}} = 555\text{-}620$ nm; for Ir(dfppy)₂⁺, $\lambda_{\text{ex}} = 405$ nm, $\lambda_{\text{em}} = 450\text{-}490$ nm). TEM images of ultrathin sections of HeLa cells incubated with LNP_{dePEG-FA} for 0.5 h (C) and 2 h (D) at 37 °C. Red arrows indicate nanoparticles at different time points.

Nuclear entry of the nanoparticles *via* the nucleopore

Flow cytometry was then applied for analysis of FA targeting effect of the nanoparticle LNP_{dePEG-FA}. The results showed that cellular uptake of LNP_{dePEG-FA} was significantly inhibited when free FA was added to the culture medium (Fig. S9 in SI). This result indicated that the cellular uptake of LNP_{dePEG-FA} was mediated *via* FA-FA receptor interaction and thus confirmed the targeting ability of FA moiety from LNP_{dePEG-FA}. The nucleopore is a channel complex of proteins which can allow access of nanoparticles up to 50 nm [9a,12]. The small sizes of the dissociated nanoparticles (*i.e.* SNP and SNP_{PEG-FA}) due to the aforementioned endo/lysosome-promoted dissociation make the nuclear entry of SNP and SNP_{PEG-FA} *via* the nucleopore possible. As mentioned before, after entry into the cytosol, most of the PEG shield detached from SNP and the oligo-*L*-lysine segments were exposed (Fig. 2A) while SNP_{PEG-FA} was still fully shielded by the PEG chains on the surface since the PEG chains in SNP_{PEG-FA} cannot be detached (Fig. 2B). To investigate the impact of PEG chains on the nuclear entry of the nanoparticles, LNP_{dePEG-FA} and LNP_{PEG-FA} were separately incubated with HeLa cells. As shown in Fig. 3A, after 6 h incubation, an intense luminescence from LNP_{dePEG-FA} appeared in the nuclear regions of HeLa cells (Fig. S5 A in SI). Besides, most of the PEG chains detached from the nanoparticles and were sequestered within the endo/lysosomes as confirmed by the co-localization of a commercial endo/lysosomal stain Lyso Tracker Deep Red® (red). The

significantly high overlap ratio ($> 70\%$) between PEG chains and the endo/lysosomes confirmed that PEG detachment took place within the endo/lysosomes. In contrast, LNP_{PEG-FA} was only sequestered in the cytosol even after 12 h incubation with HeLa cells (Fig. S4 in SI).

The inability of $[\text{Ir}(\text{dfppy})_2\mu\text{-Cl}]_2$ to enter the nucleus excludes the possibility that unreacted $[\text{Ir}(\text{dfppy})_2\mu\text{-Cl}]_2$ molecules encapsulated in the particles would be able to diffuse out and translocate into the nucleus [24h]. Furthermore, the PEG chains on the surface of LNP_{PEG-FA} appeared to inhibit the nuclear entry of the nanoparticles. This indicates the importance of the exposed oligo-*L*-lysine outside the nanoparticles for nuclear entry. Taken together, these results indicate that the native drug $[\text{Ir}(\text{dfppy})_2\mu\text{-Cl}]_2$ was not capable of entering the nucleus unassisted but could be delivered into the nucleus *via* encapsulation within the LNP_{dePEG-FA} nanoparticles. This was also confirmed by dual-channel luminescence imaging, where oligo-*L*-lysine was tagged with the fluorophore RBITC (Section 7 in SI for experimental details). After 6 h incubation, there was *ca.* 84% of overlap between RBITC (orange) and $\text{Ir}(\text{dfppy})_2^+$ (cyan) in regions of the nucleus (Fig. 3B and Fig. S5B in SI). According to the confocal fluorescent microscopy observations, SNP entered the nucleus of nearly all HeLa cells after 6 h incubation and no cell mitosis was observed during the observations. It is known that the mitosis period for HeLa cells is *ca.* 21 h [30]. If the nuclear entry had occurred during cell division, the nanoparticles would not have entered the nuclei of all the HeLa cells after the 6 h incubation. Therefore, it can be concluded that SNP entered the nucleus *via* the nucleopore. To further demonstrate this, ultrathin sections of HeLa cells incubated with LNP_{dePEG-FA} for 6 h were imaged by TEM. As shown in Fig. 3C, the nuclear entry of the nanoparticles *via* the nucleopore (as indicated by red arrows) and the nuclear localization (as indicated by red dashed circles) of the nanoparticles were observed. Some nanoparticles were even located within the nucleolus after incubation (as indicated by red dashed circles in the right panel in Fig. 3C), which may have resulted from the interaction between the oligo-*L*-Lys of SNP and intranuclear DNA.

Intranuclear drug release of the nanoparticles

As shown in Fig. 4A, after further prolonging the incubation time of HeLa cells with LNP_{dePEG-FA} to 7 h, only 60% of overlap between the luminescence of oligo-*L*-lysine (orange) and $\text{Ir}(\text{dfppy})_2^+$ (cyan) was observed, which was significantly lower than the degree of overlap at 6 h incubation (84%, Fig. 3B). This fact indicates significant phase separation between oligo-*L*-lysine and $\text{Ir}(\text{dfppy})_2^+$ in the nucleus after 7 h incubation. As previously mentioned, most of the SNP entered the nucleus of HeLa cells after 6 h incubation and thus the phase separation took place after the nuclear entry of the nanoparticles. The phase separation suggests that the $\text{Ir}(\text{dfppy})_2^+$ moieties

were significantly released from SNP into the nucleus. The intranuclear drug release was further confirmed by the real-time observation of the intranuclear nanoparticles, as recorded by confocal fluorescent microscopy in 10-min intervals for 40 min after 6 h incubation. It was found that the luminescence intensity of $\text{Ir}(\text{dfppy})_2^+$ was progressively enhanced over time (red line in Fig. 4B). Meanwhile, the overlap ratio between $\text{Ir}(\text{dfppy})_2^+$ and oligo-*L*-lysine intranuclearly decreased gradually (blue line in Fig. 4B).

Fig. 3 (A-B) Confocal luminescence images of HeLa cells incubated with $\text{LNP}_{\text{dePEG-FA}}$ (Ir: 2.0 mg/L) for 6 h with PEG tagged with RBITC (A) and oligo-*L*-lysine tagged with RBITC (B), respectively (for RBITC: $\lambda_{\text{ex}} = 543 \text{ nm}$, $\lambda_{\text{em}} = 555\text{--}620 \text{ nm}$; for $\text{Ir}(\text{dfppy})_2^+$, $\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 450\text{--}490 \text{ nm}$; for Dio, $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 500\text{--}550 \text{ nm}$; For Lyso Tracker Deep Red, $\lambda_{\text{ex}} = 633 \text{ nm}$, $\lambda_{\text{em}} = 660\text{--}750 \text{ nm}$). The white scale bars represent 10 μm . (C) TEM images of ultrathin sections of HeLa cells incubated with $\text{LNP}_{\text{dePEG-FA}}$ for 6 h, showing that SNP enters the nucleus *via* nucleopores (indicated by the red arrows) and the nuclear localization of SNP (indicated by the red dashed circles). The pink arrow indicates a nuclear pore complex (NPC).

Through screening the interactions between the Ir(III) complex and 20 different natural amino acids *in vitro*, our and other previous reports showed that only histidine can interact with $\text{Ir}(\text{dfppy})_2^+$ due to the relatively strong interaction between the imidazolyl groups and $\text{Ir}(\text{dfppy})_2^+$, which resulted in a significant enhancement in luminescence emission [19]. Thus, the luminescence enhancement in the real-time luminescence observation suggests that $\text{Ir}(\text{dfppy})_2^+$ interacted with histidine residues in the nucleus. To confirm this hypothesis, calf thymus DNA (*ct*-DNA) and nuclear protein extracts of HeLa cells were incubated with SNP. Ethidium bromide (EB) was used as a fluorescent dye for DNA [31]. It was found that the fluorescence intensity increments of EB in EB/*ct*-DNA/SNP mixtures were significantly lower than those in EB/*ct*-DNA (Fig. S6 in SI), indicating prohibition of the EB insertion by SNP. Thus SNP interacted with and possibly wrapped around DNA, which made the EB insertion difficult [32]. The results of control experiments exhibit that the fluorescent intensity of SNP was significantly enhanced in the

mixture of SNP/*ct*-DNA/nuclear proteins, while it was only slightly enhanced in either the mixture of SNP/*ct*-DNA or the mixture of SNP/nuclear proteins (Fig. S7 in SI). Therefore, the coexistence of SNP with both DNA and nuclear proteins is necessary for the remarkable fluorescence enhancement, which is consistent with the abovementioned fact that the luminescence intensity of Ir(dfppy)₂⁺ was increased significantly in the nucleus since all the components coexist in the nucleus. Considering the fact that the imidazolyl groups can interact strongly with and thus increase significantly the luminescence intensity of Ir(dfppy)₂⁺ [19], we believe that, in either the mixture of SNP/*ct*-DNA/nuclear proteins or the nucleus, the Ir(dfppy)₂⁺ moieties interact with the nuclear proteins containing imidazolyl groups of histidine residues. This interaction between the Ir(dfppy)₂⁺ moieties and nuclear proteins should be assisted by the interaction of SNP with DNA, since it does not occur considerably in the mixture of SNP/nuclear proteins (indicated by slight increase in the luminescence intensity); the interaction between DNA and SNPs was confirmed by the abovementioned EB insertion experiment. The interaction between the Ir(dfppy)₂⁺ moieties and the nuclear proteins assisted by the SNP/DNA interaction might account for the intranuclear release of the Ir(III) complex.

Intranuclear drug release inducing apoptosis of the cancer cells

To evaluate the anticancer activity of LNP_{dePEG-FA}, flow cytometric experiments were applied for analysis of HeLa cells incubated with LNP_{dePEG-FA}. In the experiments, the HeLa cells were co-stained for viability and it was found that the apoptosis ratios of the cells increased with increasing the incubation time (Fig. S8 in SI). Caspase-3 is the executor of cell apoptosis and thus ELISA to determine the levels of procaspase-3 in HeLa cells was used to investigate the apoptosis in greater details. As shown in Fig. 5A, with increasing the incubation time, higher consumption levels of procaspase-3 were observed. This confirmed that procaspase-3 was converted to caspase-3, which mediated the apoptosis of HeLa cells after LNP_{dePEG-FA} incubation [33,34].

Fig. 4 (A) Confocal luminescence images of HeLa cells incubated with $\text{LNP}_{\text{dePEG-FA}}$ (Ir: 2.0 mg/L) for 7 h; in the $\text{LNP}_{\text{dePEG-FA}}$, oligo-*L*-lysine was tagged by RBITC (RBITC: $\lambda_{\text{ex}} = 543 \text{ nm}$, $\lambda_{\text{em}} = 555\text{-}620 \text{ nm}$; Ir(dfppy)_2^+ , $\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 450\text{-}490 \text{ nm}$; Dio, $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 500\text{-}550 \text{ nm}$). (B) Time courses of luminescence intensity of Ir(dfppy)_2^+ and a control background point and overlap ratios between Ir(dfppy)_2^+ and oligo-*L*-lysine in the nucleus, as recorded by the real-time observation of intranuclear nanoparticles at 10-min intervals for 40 min after 6 h incubation. The white scale bars represent 10 μm .

MTT assay was performed on HeLa cells to evaluate cytotoxicity of the different drug formulations. The native drug $[\text{Ir(dfppy)}_2\mu\text{-Cl}]_2$ (F-Ir) was pre-dissolved in DMSO to enhance the water-solubility of the drug and determined with an IC_{50} value of *ca.* 70 μM of Ir (Fig. 5B). Notably, compared to the native drug, the nucleus-targeting $\text{LNP}_{\text{dePEG-FA}}$ showed a much lower (*ca.* 20-fold lower) IC_{50} value (Fig. 5B). Such a significant difference in cytotoxicity between $\text{LNP}_{\text{dePEG-FA}}$ and $[\text{Ir(dfppy)}_2\mu\text{-Cl}]_2$ could be attributed to the intranuclear delivery and release of the drug. $\text{LNP}_{\text{dePEG-FA}}$, with non-detachable PEG chains and encapsulated Ir(dfppy)_2^+ complex, exhibited much lower cytotoxicity compared to the native drug, suggesting the protection effects from drug release by the drug-carrier. Clearly, the intranuclear release of the metallodrug provides a highly effective way to kill HeLa cells.

Fig. 5 (A) Procaspase-3 contents determination by ELISA assay after incubating HeLa cells with LNP_{dePEG-FA} (Ir: 2.0 mg/L) for 0, 3, 6, 9, and 12 h at 37 °C. (B) Results of MTT assay for 48 h of LNP_{dePEG-FA}, LNP_{PEG-FA} and the metallodrug [Ir(dfppy)₂μ-Cl]₂ on HeLa cells. (Data are mean ± SD, n = 3, *p < 0.05).

***In vivo* tumoral uptake and nucleus-targeted drug delivery**

For the *in vivo* studies, an A549-cell-xenografted female nude mouse model was established and the mice received an intravenous injection of different drug formulations. As a control, the nanoparticles of Lys₃₀/Ir(III) complex were prepared under the same conditions and through the same processes as those for LNP_{dePEG-FA} except that oligo-*L*-Lys (Lys₃₀) was used in place of Lys₃₀-CH=N-PEG-NH₂. The as-prepared Lys₃₀/Ir(III) nanoparticles are spherical large compound nanoparticles comparable to LNP_{dePEG-FA} in size, morphology and inner structure, but without the PEG decoration [20c]. The *in vivo* uptake of nanoparticles by tumor tissues as well as by other organs was investigated and determined by ICP-MS. Compared to Lys₃₀/Ir(III) nanoparticles, which had no PEG protection, LNP_{dePEG-FA} or LNP_{PEG-FA} was significantly accumulated at the tumor sites (Fig. 6A). ICP-MS was used to monitor blood clearance of the nanoparticles. The results showed that Lys₃₀/Ir(III) nanoparticles were rapidly cleared from the bloodstream, whereas LNP_{dePEG-FA} or LNP_{PEG-FA} with PEG protection from non-specific interactions exhibited a much longer blood circulation time (Fig. S10 in SI). The prolonged blood circulation time of LNP_{dePEG-FA} or LNP_{PEG-FA} possibly promoted the nanoparticles to extravasate from the tumor blood vessels into the tumor tissues *via* the EPR effect [18,35]. It has to be mentioned here that,

during the *in vivo* application, the benzoic imine bond may rupture and PEG chains would thus detach in the acidic tumor extracellular microenvironment (pH 6.4~6.8) since the imine bond ruptures at pH 6.5 [25]. This should promote the cellular uptake of the nanoparticles and thus benefit the cancer therapy.

Fig. 6 (A) *In vivo* uptake of Lys₃₀/Ir(III) nanoparticles, LNP_{PEG-FA} or LNP_{dePEG-FA} by tumor tissues and other organs after 24 h of a single injection at a dose of 2.0 mg/kg of Ir equivalent (n = 3, data are mean $\pm$ SD, *p < 0.05). (B) Anti-tumor efficacy and (C) life-span monitoring tests of different drug formulations at a dose of 10 mg/kg of Ir equivalents for five times, as indicated by the black arrows. Tumors were measured every second day and the tumor volume was calculated accordingly (n = 8, data are mean $\pm$ SD, *p < 0.01).

The *in vivo* anti-tumor efficacy of LNP_{dePEG-FA} was further assessed on the mouse model (Fig. 6B and 6C and Section 12 in SI for experimental details). The tumor growth of mice treated with

LNP_{dePEG-FA} was much slower than that of mice treated with LNP_{PEG-FA}, Lys₃₀/Ir(III) nanoparticles or the native drug [Ir(dfppy)₂μ-Cl]₂, and the difference became significant ($p < 0.05$) after day 17. Tumor-inhibition rate (TIR) was then calculated based on the tumor volumes using a previously reported equation [36]. A 70 % of TIR was found in mice treated with LNP_{dePEG-FA}, compared with TIRs of 19, 29 and 44 % for [Ir(dfppy)₂μ-Cl]₂, Lys₃₀/Ir(III) nanoparticles and LNP_{PEG-FA}, respectively (Fig. 6B). The greater tumor inhibition achieved by LNP_{dePEG-FA} could be attributed to the enhanced tumoral uptake and nuclear delivery of the potential anticancer drug. More importantly, mice treated with LNP_{dePEG-FA} showed notably greater life-span enhancement compared to other groups (Fig. 6C). Furthermore, the mice treated with LNP_{dePEG-FA} showed no body loss during the treatment (Fig. S11 in SI). These suggest that LNP_{dePEG-FA} might function as a novel nano-platform for chemotherapy with limited physiological toxicity [18].

The antitumor mechanism of the nucleus-targeted metallodrug delivery of LNP_{dePEG-FA} was further analyzed by histological examinations. As determined by hematoxylin/eosin (H&E) staining, the tumor cells in mice treated with 0.9% NaCl, [Ir(dfppy)₂μ-Cl]₂ or Lys₃₀/Ir(III) nanoparticles (Fig. 7A-C and the insets) showed relatively full and complete cytoplasm or nuclei, while the tumor cells of mice treated with LNP_{PEG-FA} and LNP_{dePEG-FA} showed significant nuclear cavitation (Fig. 7D and the inset) and condensation (Fig. 7E and the inset), respectively. Deformation of the nucleus is a typical sign of apoptotic cells [18,37]. Indeed, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining revealed significant apoptosis of the tumor cells treated with LNP_{PEG-FA} or LNP_{dePEG-FA} (Fig. 7D' or 7E' and the insets). In comparison, the tumor cells treated with other drug formulations were negative in the TUNEL assay (Fig. 7A'~C'). These results suggest that the tumor suppression *in vivo* by LNP_{dePEG-FA} was achieved by eliciting apoptosis of the tumor cells, which was consistent with the results from the *in vitro* cell studies.

Fig. 7. Light microscopy images of (A-E) hematoxylin/eosin (H&E) and (A'-E') TdT-mediated dUTP nick end labeling (TUNEL) staining of tumor sections of the mice after treatments. In the pictures A-E, the assay was used to characterize the morphologies of

the cytoplasm and the nucleus. In (A'-E'), the assay was used to determine the apoptotic cells which are identified by positive TUNEL staining (brown) under light microscopy. The scale bar represents 200 μm .

Conclusions

We have prepared and demonstrated the large compound nanoparticle ($\text{LNP}_{\text{dePEG-FA}}$) of PEG-benzoic imine-oligo-*L*-lysine/iridium(III) metallodrug complex as a nanosystem for nucleus-targeted drug delivery *in vitro* and *in vivo*. $\text{LNP}_{\text{dePEG-FA}}$ simultaneously addresses the contradictory requirements for size and surface property of nuclear entry vehicles from blood to the intracellular nucleus. Initially, the large size of $\text{LNP}_{\text{dePEG-FA}}$ helps to avoid systematic toxicity of the nanoparticle. However, the large nanoparticle dissociates into smaller entities when they are endocytosed into acidic endo/lysosomes. The PEG chains initially bound to the large particle protect the nanoparticle from non-specific interactions in the vascular environment, but they become detached in the acidic interior of endo/lysosomes. After dissociation and PEG detachment of $\text{LNP}_{\text{dePEG-FA}}$, the resultant smaller nanoparticles can actively translocate into the nucleus *via* the nucleopore. $\text{LNP}_{\text{dePEG-FA}}$ showed *ca.* 20-fold higher cytotoxicity *in vitro* and greater tumor suppression in a mice model compared to the native drug. We believe that this work would stimulate a wide array of nanosystems directed toward *in vivo* nucleus-targeted drug delivery.

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Supplementary Data synthesis methods and NMR characterization data for the compounds; preparation and characterization of the nanoparticles; confocal microscopy images of nuclear entry of the nanoparticles; molecular analysis of apoptotic cells *in vitro*; *in vivo* blood circulation time and changes of mice body weights during the treatment.

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