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Intraperitoneal delivery of platinum with in-situ crosslinkable hyaluronic acid gel for local therapy of ovarian cancer

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Abstract

Intraperitoneal (IP) chemotherapy is a promising post-surgical therapy of solid carcinomas confined within the peritoneal cavity, with potential benefits in locoregional and systemic management of residual tumors. In this study, we intended to increase local retention of platinum in the peritoneal cavity over a prolonged period of time using a nanoparticle form of platinum and an in-situ crosslinkable hyaluronic acid gel. Hyaluronic acid was chosen as a carrier due to the biocompatibility and biodegradability. We confirmed a sustained release of platinum from the nanoparticles (PtNPs) and nanoparticle/gel hybrid (PtNP/gel), receptor-mediated endocytosis of PtNPs, and retention of the gel in the peritoneal cavity over 4 weeks--conditions desirable for a prolonged local delivery of platinum. However, PtNPs and PtNP/gel did not show a greater anti-tumor efficacy than CDDP solution administered at the same dose but rather caused a slight increase in tumor burdens at later time points, which suggests a potential involvement of empty carriers and degradation products in the growth of residual tumors. This study alerts that although several materials considered biocompatible and safe are used as drug carriers, they may have unwanted biological effects on the residual targets once the drug is exhausted; therefore, more attention should be paid to the selection of the drug carriers.

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Keywords

Intraperitoneal chemotherapy; drug delivery; hyaluronic acid; platinum; ovarian cancer; empty carriers

1. Introduction

Intraperitoneal (IP) chemotherapy has been pursued as a promising post-surgical therapy of solid carcinomas confined within the peritoneal cavity, such as ovarian cancer (OC) and peritoneal carcinomatosis. The benefit of IP chemotherapy is multifaceted. A drug delivered IP can achieve a higher concentration and a longer half-life in the peritoneal cavity compared to those observed with intravenous (IV) administration [1–3] and, thus, has a greater opportunity for locoregional effects [4–6]. Moreover, the IP-administered drug is partly absorbed to systemic circulation, getting access to regions of a tumor that are not in direct contact with peritoneal fluid or tumors remote from the peritoneal cavity via blood [7, 8]. The systemic absorption of an IP-administered drug occurs more slowly than IV drug; therefore, IP administration also brings the benefits of sustained drug delivery (a prolonged blood half-life and lower $C_{\max}$) [9–11]. Finally, if delivered as nanoparticles with a specific size, a drug can be trafficked through lymph nodes [3, 12, 13], which provides an opportunity to treat cancer cells spreading via the lymphatics.

With increasing awareness of the potential benefits, several drugs have been delivered IP for the therapy of peritoneal malignancies [14–16]; however, most of them are simple repurposing of IV drugs, not necessarily designed with special constraints for IP delivery in mind. Those requirements include (i) the biocompatibility of the material system—an important feature given the sensitivity of the peritoneal cavity to foreign insults [17], (ii) an optimum rate of degradation and absorption for an extended retention in the peritoneal cavity, and (iii) the ability to control the drug release for prolonging the local effect and attenuating systemic drug absorption. Considering these needs, we have used an in-situ crosslinkable hydrogel based on hyaluronic acid (HA) derivatives as a drug carrier to the peritoneal cavity [18–21]. The gel is composed of adipic dihydrazide modified HA (HA-ADH) and oxidized HA (HA-CHO), which can be applied as liquid and instantly crosslink via the hydrazone link to form a gel as they mix. Of several advantages of the HA gel, relevant to the IP therapy are the biodegradability, biocompatibility, and the in-situ crosslinkability, which allows for flexible and broad coverage of the peritoneal cavity [22, 23]. Moreover, HA has abundant carboxyl groups, which can form a complex with platinum (Pt) [24] and attenuate its release over several days [25–27].

Based on these advantages, we hypothesize that the in-situ crosslinkable HA gel as a carrier of Pt will localize and release the drug in the peritoneal cavity over a prolonged period, thereby more effectively reducing the tumor burden than a free drug solution. Pt compounds are an important arsenal in post-surgical chemotherapy of OC but they present significant systemic side effects such as nephro- and neurotoxicity [28, 29]; therefore, a new local delivery system for Pt compounds is well justified. Here, we prepare a nanoparticle (NP) form of Pt and deliver the NP with the in-situ crosslinkable HA gel IP (Fig. 1) to test the

utility of the Pt gel system in the local chemotherapy of tumors established in the peritoneal cavity.

2. Materials and Methods

2.1. Materials

Hyaluronic acid (HA, 35 kDa) was purchased from Lifecore Biomedical, LLC (Chaska, MN, USA). FPR-648 dye was a gift from BioActs (Incheon, Korea). Cell culture medium and supplements were purchased from Invitrogen (Carlsbad, CA, USA). All other reagents including cis-dichloro-diamine-platinum (CDDP, cisplatin) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation and characterization of Pt-incorporated HA nanoparticles (PtNPs)

PtNPs were produced as described in the literature [25–27]. Briefly, 25 mg HA and 5 mg CDDP were dissolved in 5 mL of deionized water. The mixture was stirred gently for 4 days in darkness. Non-encapsulated CDDP was removed by dialysis (molecular weight cut-off: 3.5 kDa) against deionized water for 1 day. The purified PtNPs were lyophilized for 2 days with trehalose as a lyoprotectant. For determination of Pt content, PtNPs were dissolved in 0.3 M NaCl at 37°C for 3 days [30] and analyzed with the Atomic Absorption Spectrometry (AAS) using a Perkin-Elmer 3110 Spectrometer (Waltham, MA, USA) equipped with a Pt lumina hollow cathode lamp (Perkin-Elmer). The Pt content and encapsulation efficiency were calculated as the weight ratio of Pt to PtNPs and the ratio of the measured Pt content to theoretical Pt content, respectively. The particle size and zeta potential of PtNPs were measured with Malvern Zetasizer Nano ZS90 (Worcestershire, UK).

2.3. Preparation of in-situ crosslinkable HA gel loaded with PtNPs (PtNP/gel)

In-situ crosslinkable HA derivatives, HA-ADH and HA-CHO, and a crosslinked HA gel were prepared as described previously [21]. PtNP/gel was prepared by suspending PtNPs in solutions of HA derivatives in PBS and extruding them through a common outlet using a double-barreled syringe. The HA concentration in gel was 40 mg/mL unless specified otherwise.

2.4. In vitro release kinetics

20 or 40 mg/mL of HA derivative solutions containing PtNPs were co-extruded into a Float-A-Lyzer® bag (1 mL capacity, Spectra/Por®). The bag was perforated with a 27 gauge needle 12 times. In the case of PtNPs or CDDP, the samples were suspended in PBS and loaded in a dialysis bag. In all cases, 1 mL of sample contained 0.65 mg Pt. Each bag was placed in 15 mL of PBS or PBS containing 10 U/mL hyaluronidase (HAse) and incubated at 37 °C for 5 days under constant agitation. At regular intervals, 5 mL of the release medium was sampled for AAS analysis and replaced with fresh PBS.

2.5. Cytotoxicity of PtHA NPs

SKOV3 human ovarian cancer cells (ATCC) were maintained in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were plated in a 96-well plate at a density of 10,000 cells per well in 200 µL of complete medium. After 24 h, 20 µL

of the concentrated PtNP suspension or CDDP solution was added to each well to provide Pt in the final concentration ranging from 0.065 to 65 $\mu\text{g/mL}$. A control group was treated with 20 μL of PBS. Cells were either incubated for 1 day in each treatment with a 2 day recovery period in treatment-free medium or incubated for 3 days with the treatment prior to the cell assay. The cell viability was estimated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. At the end of each treatment, the medium was replaced with 100 μL of fresh medium and 15 μL of 5 mg/mL MTT solution and incubated for 3.5 h. One hundred microliters of solubilization/stop solution was then added, and the plate was left in darkness overnight. The absorbance of the solubilized formazan was read with a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 562 nm. The measured absorbance was normalized to the absorbance of PBS-treated cells.

2.6. Confocal microscopy

Fluorescently labeled PtNPs (PtNPs*) were produced by labeling a portion of HA with a pre-activated FPR-648 dye (Ex/Em = 648/672 nm). Briefly, HA-ADH was labeled with FPR-648 according to the manufacturer's instruction and used to replace 20% of HA in PtNPs. SKOV3 cells were plated at a density of 80,000 cells/cm² in 35 mm dish with a glass window (MatTek) and incubated for 24 h. The medium was replaced with 0.3 mg/mL of PtNPs* suspended in the serum-free medium with or without 10 mg/mL of HA. After 1 h incubation, the NP containing media was replaced with the fresh serum-free media, and the dishes were washed with media twice to remove free PtNPs*. Hoechst 33342 was added to 2 $\mu\text{g/mL}$ 30 min prior to imaging to stain the nuclei. Confocal microscopy was performed using a Nikon A1R confocal microscope equipped with a Spectra Physics 163C argon ion laser and a Coherent CUBE diode laser. PtNPs* were excited with a 488 nm laser, and the emission was read from 500 to 600 nm. Cell nuclei were excited with a 633 nm laser, and the emission was read from 650 to 750 nm.

2.7. Determination of the maximum tolerated doses (MTDs) of treatments

All animal procedures were approved by Purdue Animal Care and Use Committee, in conformity with the NIH guidelines for the care and use of laboratory animals. The MTD of each treatment was determined according to the method published by the National Cancer Institute's Developmental Therapeutics Program [31]. Healthy female Balb/c wild-type mice (17–20g, Harlan Laboratories, Indianapolis, IN), were randomly assigned to CDDP, PtNP, and PtNP/gel groups and given a single IP injection of each formulation at different dose levels (one mouse per dose). The mice were observed over a period of 2 weeks after the injection. The highest dose tolerated without >20% weight loss or other signs of significant toxicity was designated as the MTD of each treatment.

2.8. Anti-tumor effectiveness of treatments in an IP tumor model

A mouse model of IP tumor was prepared as described in our previous study [21]. SKOV3 (ATCC, Manassas, VA, USA) or luciferase-expressing SKOV3 cells (SKOV3-luc, donated by Prof. Glen Kwon at University of Wisconsin-Madison) [32] were maintained in a complete RPMI-1640 medium. Ten million cells were suspended in 1 mL PBS and injected

to a female Balb/c nude mouse (8–10 week old, ~20g, Harlan Laboratories). Two sets of experiments were performed independently.

In the first set (Supporting Fig. 1a), animals were injected with SKOV3 cells 2 weeks before the treatment. Animals were randomly assigned to three Pt formulations (CDDP, PtNPs, PtNP/gel: 4.8 mg Pt/kg as a single dose) and two negative control treatments (PBS and blank gel) (n=10 per group). The treatments were administered as 1 mL IP injection. At 2 or 4 weeks after the treatment, 5 animals per group were sacrificed by ketamine anesthesia followed by cervical dislocation, and the weight and location of tumors in the peritoneal cavity were recorded. Blood was collected by cardiac puncture and submitted to Animal Disease and Diagnostic Laboratory for blood chemistry analysis. Peritoneal lavage samples were collected as previously described [21], and the level of Pt remaining in the lavage fluid was determined by AAS.

In the second set (Supporting Fig. 1b), animals were injected with SKOV3-luc cells, and tumor burdens were monitored weekly using the whole body imaging system. Three weeks later, animals with different tumor burdens were evenly assigned to five groups (n=3–4 per group) and administered with treatments at a total dose of 7.5 mg Pt/kg (CDDP, PtNP: 3.8 mg Pt/kg twice with a week interval; PtNP/gel: 7.5 mg Pt/kg once). Bioluminescence of IP tumors was measured weekly over 5 weeks. After the final imaging, animals were sacrificed to locate tumors and determine the weights. Throughout the duration of experiments, animals were observed every other day for weight change and signs of pain.

2.9. Whole body imaging

Tumor growth was monitored by the IVIS Lumina II whole body imaging system (Caliper Life Science, Hopkinton, MA, USA). Briefly, animals were anesthetized by 2% isoflurane, and D-luciferin (Gold Biotechnology, St. Louis, MO, USA) was injected IP into the tumor-bearing mouse approximately 5 min prior to whole-body imaging. All images were collected using identical system settings (exposure time: 120s; binning: medium; f/stop: 2) over 16 min. Living Image® software was used to quantify the intensity of bioluminescence signal (expressed in radiance) from SKOV3-luc tumors, and the maximum value was recorded as an estimation of tumor burden.

2.10. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 (La Jolla, CA). Unless specified otherwise, one-way ANOVA was used to determine the difference among the groups, and pairs were compared using Dunnett's multiple comparisons test. A value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Platinum release was attenuated by HA NPs

Pt was initially loaded as cis-dichloro-diamine-platinum (CDDP) in the HA hydrogel, but there was no difference in release kinetics between CDDP and CDDP encapsulated in HA gel (data not shown). This result indicates that HA gel had no direct interactions with Pt in

CDDP, thus having little effect on the Pt release. Since the HA gel did not attenuate the CDDP release, Pt was loaded in the gel as a NP form (Fig. 1). The NPs were produced by incubating HA and CDDP over 3 days to allow for carboxylates of HA to replace chlorides of CDDP and form a chelate-type coordination bond with Pt [33]. The Pt-loaded HA NP (PtNP) was expected to serve two purposes: (i) to increase the molecular mass of the Pt compound for a better retention in HA gel and (ii) to attenuate Pt release through the metal-carboxylate interactions.

The average diameter of PtNPs was 270.2 ± 33.2 nm (average and standard deviation of 4 independently prepared batches) with a polydispersity index ranging from 0.18–0.53, which indicates a mid-range polydispersity [34]. The zeta potential of PtNPs was -21.0 ± 3.1 mV (average and standard deviation of 3 independently prepared batches) at pH 7.4, which reflected the negative charge of HA, the encapsulating polymer. The average Pt content (% weight ratio of Pt to PtNPs) was 8.7 ± 0.8 wt%, corresponding to the encapsulation efficiency (% ratio of the measured Pt content to theoretical Pt content) of 80.4 ± 7.4 %. PtNPs showed a sustained release of Pt over 96 hours after 7 hours of lag time in PBS (Fig. 2a). In the presence of 10 U/mL of Hase, an enzyme present in the body, Pt release occurred with no delay and was complete in 72 hours due to accelerated HA degradation. There was no difference between PtNPs and PtNPs loaded in HA gel (PtNP/gel), irrespective of the gel concentration (20 or 40 mg/mL HA), confirming that HA gel alone did not contribute to the control of Pt release. Therefore, the main role of HA gel would be to localize PtNPs in the peritoneal cavity.

3.2. Cytotoxicity of PtNPs was consistent with the release kinetics

Due to the attenuation of drug release, PtNPs showed less cytotoxicity than CDDP when the cellular exposure was limited to one day (Fig. 2b). On the other hand, when SKOV-3 cells were exposed to PtNPs for 3 days, by which the majority of Pt would have been released, there was no difference between CDDP and PtNPs in the cytotoxic activity. The half maximal inhibitory concentrations (IC_{50}) of CDDP and PtNPs for SKOV3 cells were $3.4 \mu\text{g Pt/mL}$ (CDDP) and $9.9 \mu\text{g Pt/mL}$ (PtNPs) after 1 day exposure followed by 2 day recovery and $3.5 \mu\text{g Pt/mL}$ (CDDP) and $3.1 \mu\text{g Pt/mL}$ (PtNPs) after 3 day exposure, respectively.

3.3. PtNPs were taken up by SKOV3 cells via HA-receptor mediated endocytosis

While PtNPs are expected to remain in the HA gel, localized in the peritoneal cavity, we also envision that parts of PtNPs may be released from the degrading gel and access the tumors to deliver Pt. To predict the direct effect on tumors, cellular uptake of PtNPs was evaluated by confocal microscopy. Fluorescently labeled PtNPs (PtNPs*) were produced using HA covalently conjugated with a fluorescent dye. PtNPs* were found in the cytoplasm of SKOV3 cells after 1 hour incubation (Fig. 3). On the other hand, PtNP* uptake was not observed when the cells were incubated with excess HA prior to the PtNP treatment. The competitive inhibition of PtNP* uptake by HA, consistent with other studies of HA-based NPs [35, 36], suggests that the NPs entered cells via receptors for HA. CD44 is one of the best known receptors for HA [37], expressed on the surface of various cancer cells [38–42] including SKOV3 cells [43, 44]. This result indicates that PtNPs released during the HA gel

degradation may provide a selective effect on cancer cells overexpressing HA receptors such as CD44.

3.4. MTDs of IP-administered treatments reflected the role of HA gel as a local reservoir of PtNPs

Prior to in vivo administration, MTDs of PtNPs and PtNP/gel were determined using healthy Balb/c mice and compared with that of CDDP. Animals did not survive 6 mg Pt/kg of CDDP and PtNPs, but PtNP/gel was tolerated up to 7.5 mg Pt/kg. It is interesting that PtNPs had a similar MTD as CDDP despite the slower drug release (Fig. 2a), whereas PtNP/gel with a similar release profile as PtNPs had a relatively higher MTD. It is possible that there was a mechanism to increase the systemic absorption of PtNPs from the peritoneal cavity to counteract the sustained Pt release, which would have been delayed by the gel. A potential condition to enhance their systemic absorption include the accelerated Pt release from PtNPs due to a dilution in the peritoneal fluid or increased lymphatic trafficking via HA receptors such as Lymph Vessel Endothelial HA receptor-1 (LYVE-1) [45]. Li et al made a relevant observation, where the MTD of Pt-HA (Hyplat) particles was slightly lower than that of CDDP (5 mg Pt/kg for Hyplat vs. 6.5 mg Pt/kg for CDDP) [46]. The authors attributed this result to the increased AUC (area under the curve) and half-life of Hyplat particles in blood [46].

3.5. Anti-tumor effects of PtNPs and PtNP/gel compared unfavorably with that of CDDP

The effectiveness of IP-administered treatments was tested with nude mice bearing SKOV3 or SKOV3-luc tumors in the peritoneal cavity. In the first set of experiments, where tumors were visually inspected post-mortem, tumor weights of CDDP, PtNP, or PtNP/gel-treated groups at 2 weeks post-treatment appeared slightly less than that of the PBS-treated group, with only the PtNP group showing a statistical significance (Fig. 4a, supporting Fig. 2a). At 4 weeks post-treatment, the CDDP-treated group showed the most significant reduction in tumor weight, followed by the PtNP-treated group, but the PtNP/gel-treated group showed no statistical difference from the PBS-treated group. Interestingly, the blank gel-treated group showed a smaller tumor weight than the PBS-treated group at 2 weeks, although this difference was not seen at 4 weeks. Gel residues were present in all 10 animals receiving gel treatments (blank gel or PtNP/gel) at 2 weeks post-treatment and 8 out of 10 animals at 4 weeks (Fig. 4b). The gel residues were noticeably opaque, in contrast to the transparent original gels, indicating an increased cellularity associated with the gels. On the other hand, no Pt was detected in the lavage samples collected at 2 or 4 weeks, indicating that Pt was completely released in less than 2 weeks.

To confirm this result, we performed another set of experiments with a higher dose of Pt (7.5 mg/kg as compared to 4.8 mg/kg of the first experiment), monitoring the tumor burdens via the non-invasive imaging. Since the MTDs of CDDP and PtNP were no greater than 6 mg Pt/kg, they were administered in two half-doses (3.8 mg Pt/kg $\times$ 2) with a week interval, making the total dose equal to 7.5 mg Pt/kg. Consistent with the first experiment, the CDDP group was most effective in suppressing the tumor growth, whereas the PtNP- or PtNP/gel-treated groups displayed increasing tumor signals after an initial attenuation over 3–4 weeks (Fig. 5, Supporting Fig. 2b), although the difference was not statistically significant.

In both experiments, there was no change in kidney weight due to the treatments, indicating no appreciable nephrotoxicity by Pt treatments (Supporting Fig. 3a). Slight body weight reduction was observed at later time points in animals treated with PtNPs and PtNP/gel (Supporting Fig. 3b). There were no clinically significant abnormalities in blood chemistry (glucose, creatinine, alanine aminotransferase, and alkaline phosphatase) in any treatment groups (Supporting Fig. 4).

3.6. Potential role of HA-based carriers

The two experiments consistently show that PtNPs and PtNP/gel were not superior to CDDP in suppressing tumor growth, contrary to our hypothesis. As suggested in the MTD study, it is unlikely attributable to the initial attenuation of drug release: in the second experiment, a single dose of PtNP/gel was compared with CDDP solution administered in two half-doses with a week interval (mimicking sustained drug release) but did not achieve a comparable effect. This result is also not explained by rapid in-vivo drug release. In that case, their anti-tumor effects should have been at least comparable to that of CDDP, but PtNPs and PtNP/gel seemed to do rather poorly at later phases. Considering that the drug release was complete in less than 2 weeks but carriers remained in the peritoneal cavity, we speculate that the empty carriers (HA NPs and HA gel) and/or their degradation products may have partaken in the tumor growth. The total amounts of HA applied in the form of carriers are quite different (PtNPs: ~50 mg/kg vs. PtNP/gel: ~2050 mg/kg); however, given that the gels were not completely degraded over 4–5 weeks, the effective HA concentrations in the peritoneal fluid might have been in comparable ranges.

This speculation may be supported by the fact that HA is not biologically inert. HA is a main component of connective tissues and fluids in human body with several biological functions such as lubrication, water homeostasis, macromolecular filtering, and regulation of cellular activities [47]. HA is known as an activator of cell migration and proliferation and a regulator of inflammation and tissue remodeling; therefore, its biological activities have been explicitly exploited in the context of tissue engineering and wound healing [48]. Moreover, overproduction of HA was linked to the enhanced tumor progression [49, 50]. In IP tumors, an HA-based film (Seprafilm®), an adhesion barrier used for post-surgical adhesion prevention, was associated with an increased rate of local tumor growth [51], consistent with our observation. In addition, it has been argued that free HA interferes with interactions between tumor cells and monocytes or tumor-infiltrating macrophages, thereby suppressing their cytotoxic responses against tumors [52, 53]. We tested if HA and its monomer constituents (N-acetyl-D-glucosamine and D-glucuronic acid) augmented proliferation of SKOV-3 cells in vitro but found that they had no prominent effect (Supporting Fig. 5). This indicates that the empty carrier effect is likely a consequence of more complex interplay of materials, tumor, and host immunity rather than the material's direct effect on cancer cells. The potential roles that the empty carriers and the degradation products may have played include the enhancement of cell proliferation and tumor invasion [54], angiogenesis [55], epithelial to mesenchymal transition [56], or suppression of the innate immunity against tumors [52, 53], which is a topic that warrants further investigation.

On the other hand, it is noteworthy that in the first experiment the blank gel initially reduced the tumor burden in 2 weeks as compared to PBS. A similar observation was made in our previous study [21] and by Emoto et al [57], although the differences were not significant. A relevant argument is that exogenous HA oligomers rather inhibit tumor progression by competitively inhibiting interaction of endogenous HA polymer with CD44 on tumor cells [58]. Therefore, the effect of empty HA carriers on tumor growth may not be simply generalized. A difference between tumors apparently suppressed by the blank gel and those enhanced by the empty carriers is that the latter group had been exposed to Pt. It remains to be investigated whether a prior exposure to Pt played a role in making the residual tumors proliferate in response to the empty carriers.

While our *in vivo* studies show the ineffectiveness of PtNPs and PtNP/gel, several other studies using HA as a carrier of anti-cancer drugs conclude otherwise. For example, paclitaxel was delivered to IP tumors as a conjugate with HA via a single IP injection achieved superior anti-tumor efficacy (reduced tumor burden and prolonged survival) compared to non-treated control or multiple Taxol IP injections [35]. Similarly, an IP-administered HA-paclitaxel conjugate was found to be more effective than IP Taxol in inhibiting tumor dissemination and prolonging survival [43]. However, these studies are not directly comparable to our study in that the treatments were administered at the MTD of each formulation ($20 \text{ mg/kg} \times 4$ for Taxol vs. $42 \text{ mg/kg} \times 4$ for HA-paclitaxel in paclitaxel equivalent [43]) and that as covalent conjugates of paclitaxel and HA, the formulations would have released the anti-cancer drug at a slower rate than the PtNPs and PtNP/gel. We have also used the current gel system in IP delivery of paclitaxel in a similar manner (non-covalent encapsulation of a drug) as the current study and found that paclitaxel/gel was at least comparable to Taxol at the same dose (30 mg/kg) [21]. A main difference between that and the present studies is that paclitaxel was much less water-soluble than Pt; thus, the drug effect lasted throughout the survival period (2 weeks) [21]. There are other studies using Pt and HA in IP delivery, which conclude good anti-tumor efficacy of HA-based formulations. For example, Emoto et al used the same HA gel system to deliver CDDP and found significant reduction in tumor burdens by Gel-CDDP as compared to PBS treatment, but not by CDDP solution [57]. However, this study may not be directly compared with ours in that the treatments were administered 3 times with a week interval, thus leaving a shorter (or no) exposure to drug-free vehicles. On the other hand, Li et al delivered CDDP as encapsulated in $\sim 500 \text{ nm}$ HA particles (Hyplat) IP to animals with IP tumors in a similar dose and schedule as ours and observed a lower clearance from blood and tissues, greater tumor accumulation of Pt, and higher anti-tumor efficacy than CDDP [46]. A potential explanation for the difference from our result is that the Hyplat particles were prepared at 90°C to facilitate the chloride-carboxylate ligand replacement; thus, they might have been more stable *in vivo* [46]. Consequently, the Hyplat particles could have better taken advantage of CD44-mediated targeting effect and offset any potential effect of HA.

In summary, our results show that the HA gel system delivering Pt in a NP form did not show a greater anti-tumor efficacy as compared to a free drug solution at the same dose. The PtNP/gel as well as PtNPs even showed a slight increase in tumor burdens after Pt was exhausted, suggesting a proliferative effect of the empty carriers and degradation products on residual tumors. Although our result warrants some concern, further studies remain to be

performed before a general conclusion can be made against the use of HA as an IP drug carrier for peritoneal malignancies. In order to reach such a conclusion, future studies should confirm that carriers with alternative compositions bring about different outcomes than ours at a comparable dose and treatment schedule. Another important lesson learned in this study is that carrier materials widely known as biocompatible may not be considered biologically inert, and more attention should be paid to their effects on the residual targets after the drug is exhausted.

4. Conclusion

In an attempt to increase local retention of Pt in the peritoneal cavity over a prolonged period, thereby enhancing the local therapy of OC, Pt was delivered in the form of NPs using an in-situ crosslinkable HA gel as a medium to form a local reservoir. The PtNPs and PtNP/gel showed sustained Pt release in vitro over 3 days, and the PtNPs were taken up by receptor-mediated endocytosis. However, PtNPs and PtNP/gel did not show a greater anti-tumor efficacy than CDDP solution administered at the same dose but rather caused a slight increase in tumor burdens at later time points. This result suggests potential involvement of empty carriers and degradation products in the growth of residual tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Schematic diagram of intraperitoneal delivery of platinum with an in-situ crosslinkable hyaluronic acid gel and nanoparticles.

Fig. 2.

(a) Platinum release kinetics from CDDP, PtNPs, and PtNP/gels in PBS and PBS containing 10 U/mL HAse. Each data point represents an average value and standard deviations of 3 identically and independently prepared samples. Subscript after PtNP/gel indicates the concentration of HA in the gel in mg/mL. For example, PtNP/gel₂₀ indicates PtNPs suspended in 20 mg/mL HA gel. (b) Cytotoxicity of PtNPs and CDDP in SKOV3 cells after 1 or 3 day incubation. Data are expressed as averages and standard deviations of 3 measurements of a representative batch.

Fig. 3. Confocal microscopy of fluorescently labeled PtNPs (PtNPs*) incubated with SKOV3 cells for 1 h in the absence (HA-) or presence (HA+) of 10 mg/mL HA.

Fig. 4.

(a) Weights of IP tumors in animals treated with PBS, blank gel, CDDP, PtNPs, and PtNP/gel for 2 or 4 weeks (1st experiment). Data are expressed as median with ranges of 5 animals. The box extends from the 25th to 75th percentiles, the line in the middle of the box indicates the median, and the whiskers go down to the smallest value and up to the largest. *: $p < 0.05$; **: $p < 0.005$ vs. PBS at each time point, by Dunnett's multiple comparison. (b) PtNP/gels recovered 2 or 4 weeks after the treatment. Left: PtNP/gel on site; right: PtNP/gel separated from the abdominal organs by tweezers.

Fig. 5.

(a) Bioluminescence of IP tumors in animals treated with PBS, blank gel, CDDP, PtNPs, and PtNP/gel (2nd experiment). Data are expressed as median with ranges of 3–4 animals. The box extends from the 25th to 75th percentiles, the line in the middle of the box indicates the median, and the whiskers go down to the smallest value and up to the largest. *: $p < 0.05$ vs. PBS at each time point, by Dunnett's multiple comparison. The inset graph shows the weights of tumors harvested from the animals after the final imaging (5 weeks after the treatment). There was no difference between the PBS group and each treatment group (blank gel, CDDP, PtNPs, and PtNP/gel). (b) Whole body bioluminescence imaging of animals administered with different treatments. The most representative animal in each group is presented.