In Situ Silver-based Electrochemical Bioreactor In Vivo

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Article

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Abstract

In this study we show for the first time that a reduced graphene oxide (rGO) carrier has a 15-fold higher catalysis rate than graphene oxide (GO) in Ag\(^+\) reduction. Based on this, we constructed a tumor microenvironment-enabled \textit{in situ} silver-based electrochemical oncolytic bioreactor (SEOB) which unlocked an Ag\(^+\) prodrug to generate silver nanoparticles and inhibited the growth of various tumors. In this bioreactor system, intratumoral H\(_2\)O\(_2\) acted as the reductant and the rGO carrier acted as the catalyst. Chelation of aptamers to this prodrug increased the production of silver nanoparticles by tumor cells, especially in the presence of Vitamin C, which broke down in tumor cells to supply massive amounts of H\(_2\)O\(_2\). Consequently, highly efficient silver nanoparticle-induced apoptosis was observed in HepG2 and A549 cells \textit{in vitro} and in HepG2- and A549-derived tumors \textit{in vivo}. The apoptosis was associated with ROS-induced changes in mitochondrial membrane potential and DNA damage. The specific aptamer targeting and intratumoral silver nanoparticle production guaranteed excellent biosafety, with no damage to normal cells, because the Ag\(^+\) prodrug was specifically unlocked in tumors. More significantly, there was no evident tissue damage in monkeys, which greatly increases the clinical translation potential of the SEOB system.

Introduction

Since the concept of nanomedicine came into existence\(^1\)\(^-\)\(^3\), various nanomaterials have been developed to execute diagnosis or treatment of malignant tumors\(^4\)\(^-\)\(^8\). However, the unsatisfactory delivery efficiency into tumors and universal distribution across all organs remain unresolved and intractable challenges even in the presence of active targeting. Enlightened by a microbial medicine factory\(^9\),\(^10\), a concept has emerged in which a biological body can act as a bioreactor for \textit{in situ} production of functional nanoagents \textit{via} chemical reaction or physical assembly of injected precursors. The specific species and microenvironment within the biological body can act as catalysts, stimuli or reactants\(^11\),\(^12\). This biosynthesis technology holds great potential for overcoming the systemic toxicity and low delivery efficiency that are associated with current drug delivery systems\(^13\). Nevertheless, this technology is still at its infancy, especially for biosynthesis of anti-tumor nanomaterials. Herein, inspired by intriguing reports highlighting H\(_2\)O\(_2\) as a reductant\(^14\),\(^15\), we developed a tumor microenvironment-enabled \textit{in situ} silver-based electrochemical oncolytic bioreactor (SEOB) to unlock an Ag\(^+\) prodrug, which establishes the intratumoral silver-based nanosynthetic medicine. The underlying principle of the SEOB theory is illustrated in Fig. 1a.

In this unprecedented SEOB theory, intratumoral H\(_2\)O\(_2\) can facilitate \textit{in situ} reduction of an Ag\(^+\)-DNA conjugate prodrug loaded in reduced graphene oxide (rGO) nanosheets. This leads to intratumoral production of silver nanoparticles, which are regarded as excellent anti-tumor agents due to their ability to induce the production of reactive oxygen species (ROS)\(^16\),\(^17\). rGO is a well-accepted drug carrier\(^18\),\(^19\),\(^20\), which can accommodate Ag\(^+\)-DNA. In addition, rGO features few oxygen-containing groups on its
surface, and therefore benefits rapid electron transfer\textsuperscript{21-23}, which enables the rapid reduction of Ag\textsuperscript{+} by H\textsubscript{2}O\textsubscript{2} in the tumor. Notably, DNA-Ag\textsuperscript{+} conjugate prodrug is obtained via the coordination interaction, wherein DNA is highlighted to stabilize Ag\textsuperscript{+}, manipulate Ag\textsuperscript{+} loading content, avoid self-driven Ag\textsuperscript{+} nucleation and growth, improve biosafety via reducing Ag\textsuperscript{+} leakage and supply rich binding sites for the subsequent chitosan (CS) coating.

To improve the accumulation of the Ag\textsuperscript{+} prodrug-carrying bioreactor in the tumor, rGO was coated with CS, then further conjugated with aptamers (Apts) targeting proteins that are specifically overexpressed in tumor cells. Two Apts were used, i.e., AS1411 and endoglin (END), which target nucleolin and endoglin, respectively\textsuperscript{24}. Thus, we obtained two Ag\textsuperscript{+} prodrug-carrying bioreactors (represented as Apt-CS/rGO/Ag\textsuperscript{+}-DNA), namely END-CS/rGO/Ag\textsuperscript{+}-DNA and AS1411-CS/rGO/Ag\textsuperscript{+}-DNA (Fig. 1b). Our strategy is designed to deliver more Ag\textsuperscript{+}-DNA conjugates into tumors, which then generate intratumoral silver nanoparticles and further facilitate silver nanoparticle-mediated anti-tumor effects. It has been shown that that Vitamin C (VitC) can specifically kill tumor cells when it breaks down to produce H\textsubscript{2}O\textsubscript{2}\textsuperscript{25-29}. Tumor cells have low catalase activity compared to normal cells, and cannot efficiently clear H\textsubscript{2}O\textsubscript{2}. Therefore, exogenously supplemented VitC is anticipated to cause massive production of H\textsubscript{2}O\textsubscript{2} in tumor cells, which acts as a reductant to produce more silver nanoparticles, thus delivering highly efficient anti-tumor activity (Fig. 1a). More impressively, the aptamer-mediated tumor targeting and specific generation of intratumoral silver nanoparticles guarantees the excellent biosafety of this bioreactor system. The SEOB causes no evident damage to the main organs of cynomolgus monkeys, which indicates that it is suitable for clinical translation.

**Results**

**VitC-enhanced H\textsubscript{2}O\textsubscript{2} reduction assay**

We firstly investigated whether H\textsubscript{2}O\textsubscript{2} can act as the reductant, because this is an essential prerequisite to fulfil the function of the SEOB. Herein, VitC was used to increase H\textsubscript{2}O\textsubscript{2} production in HepG2 tumor cells, since several studies have reported VitC-derived H\textsubscript{2}O\textsubscript{2} elevation in tumor cells\textsuperscript{25-27}. When we treated HepG2 cells with Ag\textsuperscript{2+} alone, we observed black silver deposits due to the pre-existing H\textsubscript{2}O\textsubscript{2} in the cells (Supplementary Fig. S1). However, many more silver deposits were observed when VitC was added prior to or after Ag\textsuperscript{+} to stimulate the production of H\textsubscript{2}O\textsubscript{2}. This confirms that H\textsubscript{2}O\textsubscript{2} can indeed behave as the reductant to reduce Ag\textsuperscript{+} into silver nanoparticles. The level of silver nanoparticles increases as either the Ag\textsuperscript{+} concentration or the incubation time escalates, accompanied by more silver-induced HepG2 apoptosis (Supplementary Figs S2 and S3). To test the ability of VitC to induce H\textsubscript{2}O\textsubscript{2} production \textit{in vivo}, we used mice bearing HepG2-derived tumors. We injected mice intratumorally or intravenously with VitC, then monitored changes in the electrical current in the tumor, which reflect the H\textsubscript{2}O\textsubscript{2} level. Significantly increased current signals, representing increased H\textsubscript{2}O\textsubscript{2} levels only at the tumor site occurred after intratumoral or intravenous injection of VitC (Fig. 1c-e). This implies that VitC-derived H\textsubscript{2}O\textsubscript{2} can reduce
the Ag\textsuperscript{+}-based prodrug *in vivo*. However, silver deposition induced by H\textsubscript{2}O\textsubscript{2} alone is inadequate due to the low reduction efficiency. To address this, rGO was used as the catalyst.

**Analysis of the catalytic activity of rGO**

Despite sharing similar structures with grapheme oxide (GO) (Supplementary **Fig. S4**), rGO is preferred to promote electron separation and H\textsubscript{2}O\textsubscript{2} disproportionation due to its much higher catalytic activity\textsuperscript{21-23}. Compared to GO, rGO allows rapid electron transfer and favors free Ag\textsuperscript{+} deposition, as evidenced by the larger silver stripping current, shorter stable response time and higher H\textsubscript{2}O\textsubscript{2} disproportionation current in various anodic stripping voltammetry (ASV) tests (**Fig. 1f-h**). Based on these extraordinary features, we selected rGO to accelerate Ag\textsuperscript{+} reduction by H\textsubscript{2}O\textsubscript{2}. Furthermore, silver deposition from an Ag\textsuperscript{+}-DNA conjugate was explored *via* ASV testing. The results showed that the successful capture of Ag\textsuperscript{+} by DNA can concentrate Ag\textsuperscript{+} and generate the strongest silver nanoparticle-derived current signal (**Fig. 1i**). This implies that Ag\textsuperscript{+} chelated to DNA was efficiently reduced by H\textsubscript{2}O\textsubscript{2} to augment the silver deposition. Therefore, it is reasonable to expect that the Apt-CS/rGO/Ag\textsuperscript{+}-DNA prodrug will rapidly and efficiently produce silver nanoparticles in the presence of rGO catalyst. To understand why rGO can achieve such a high catalytic activity for silver deposition, Raman, Fourier transform infrared (FT-IR) and X-ray photoelectron spectroscopy (XPS) characterizations were carried out. Compared to GO, rGO had a higher D/G ratio and fewer oxygen-containing functional groups (Supplementary **Fig. S5**). The decreased oxygen content in rGO means that more sp\textsuperscript{2} hybrid orbitals are available for accommodating electrons. In this regard, rGO can undoubtedly increase electron transfer from disproportionated H\textsubscript{2}O\textsubscript{2} to Ag\textsuperscript{+}, thus favoring catalytic reduction of Ag\textsuperscript{+} and deposition of silver nanoparticles.

**Synthesis of a bioreactor carrying the Ag\textsuperscript{+} prodrug**

The decreased level of oxygen-containing functional groups in rGO facilitates π-π conjugation, which will enhance the binding affinity between rGO and Ag\textsuperscript{+}-conjugated DNA chains (Ag\textsuperscript{+}-DNA) and enable rGO stacking (**Fig. 2a**). When the rGO carrier is chelated with Ag\textsuperscript{+}-DNA conjugates, coated with CS and modified with AS1411 aptamers, the structure of the carrier is not changed (**Fig. 2b**). This suggests that AS1411-CS/rGO/Ag\textsuperscript{+}-DNA will retain the rGO-catalyzed silver deposition property. The presence of uniformly distributed Ag and P elements demonstrates the successful chelation of Ag\textsuperscript{+}-DNA conjugates onto rGO in AS1411-CS/rGO/Ag\textsuperscript{+}-DNA (**Fig. 2c-e**). New FT-IR characteristic peaks and changes in the zeta potential also demonstrate the successful synthesis of AS1411-CS/rGO/Ag\textsuperscript{+}-DNA and its intermediate products in sequence (**Fig. 2f,g**). During the modification process, the particle size remains approximately constant (Supplementary **Fig. S6a**).

**Aptamer-mediated targeting assay *in vitro***

AS1411 specifically targets nucleolin, which is overexpressed by many tumors\textsuperscript{30-32}. Therefore, we investigated the internalization of AS1411-CS/rGO/Ag\textsuperscript{+}-DNA into HepG2 cells, which overexpress
nucleolin. Direct analyses by laser confocal scanning microscopy (LCSM) and flow cytometry were firstly performed. A random sequence (RS) incapable of tumor targeting was used instead of AS1411 to synthesize the control nanosystem (i.e., RS-CS/rGO/Ag+-DNA). Furthermore, the L02 cell line, which features low nucleolin expression, was used as another comparison. The results clearly showed that more AS1411-CS/rGO/Ag+-DNA than RS-CS/rGO/Ag+-DNA entered the HepG2 cells, and both systems failed to enter L02 cells (Fig. 2h). This result sufficiently validates the specific targeting of AS1411 to nucleolin-overexpressing HepG2 cells. Flow cytometry data also confirms that there is more accumulation of AS1411-CS/rGO/Ag+-DNA in HepG2 cells than RS-CS/rGO/Ag+-DNA (Supplementary Fig. S7).

SEOB unlocks the Ag+ prodrug for silver deposition and anti-tumor activity

Contributed by AS1411 targeting-enhanced AS1411-CS/rGO/Ag+-DNA accumulation, VitC-enhanced H2O2 production and rGO-catalyzed silver deposition, AS1411-CS/rGO/Ag+-DNA+VitC group exerts the most robust killing ability against HepG2 cells. (Fig. 2i) This phenomenon attributed to that silver nanoparticles birth in the AS1411-CS/rGO/Ag+-DNA+VitC group gave birth to more oxidative stress for altering or preventing cell cycle progression. However, the targeting ability of AS1411 determines AS1411-CS/rGO/Ag+-DNA failed to induce evident injures to normal liver cells with low nucleolin expression (e.g., L02) (Fig. 2k). Intriguingly, VitC-enhanced H2O2 production alone does not kill HepG2 and L02 cells (Fig. 2j), and few AS1411-CS/rGO/Ag+-DNA and RS-CS/rGO/Ag+-DNA nanoparticles accumulate in L02 cells because these systems are not specifically targeted to L02 (Fig. 2k,l). These impressive results (i.e. assessment of the levels of silver deposition and cell apoptosis) indirectly validate the targeting ability of AS1411. More significantly, laser confocal scanning microscopy (LCSM) observations of HepG2 cells after live/dead co-staining also demonstrate that AS1411-CS/rGO/Ag+-DNA+VitC induces the most cell apoptosis and results in the lowest cell density (Fig. 2m).

In-depth exploration of the anti-tumor effect of AS1411-CS/rGO/Ag+-DNA+VitC was carried out via monitoring the variation of mitochondrial membrane potential. The strongest green fluorescence of JC-1 monomers was observed in AS1411-CS/rGO/Ag+-DNA+VitC-treated HepG2 cells (Fig. 3a). This reflects the significantly decreased membrane potential in these cells, and suggests a high level of apoptosis. In contrast, the negligible level of green fluorescence in L02 cells indicates no change in membrane potential and no L02 apoptosis, which can be attributed to poor accumulation of AS1411-CS/rGO/Ag+-DNA in L02 cells (Fig. 3b). These results adequately demonstrate that AS1411-CS/rGO/Ag+-DNA in the presence of VitC can produce the most silver deposits, which triggers the greatest anti-tumor activity. Quantitative data also confirms that although VitC-enhanced H2O2 alone is safe, VitC in combination with AS1411-CS/rGO/Ag+-DNA can induce the generation of more silver nanoparticles to robustly inhibit the growth of HepG2 cells (Fig. 3c,d). Moreover, this combination has only weak effects on L02 cells (Fig. 3d). Similar results were obtained via evaluating DNA damage. AS1411-CS/rGO/Ag+-DNA+VitC treatment causes the longest tail in HepG2 cells, which means the highest degree of DNA damage (Fig. 3e,f). However, AS1411-CS/rGO/Ag+-DNA+VitC fails to induce evident apoptosis in L02 cells due to the low
accumulation of AS1411-CS/rGO/Ag⁺-DNA in these cells (Fig. 3g,h). This tumor cell-specific effect guarantees the safety of the treatment. Furthermore, we also measured ROS levels, since ROS are directly responsible for apoptosis and ROS levels positively correlate with the deposition of silver nanoparticles. VitC alone is unable to generate sufficient ROS-induced oxidative stress for inducing HepG2 apoptosis even though the VitC concentration reached a high level (above 8 mM) (Fig. 3i). Once VitC is combined with AS1411-CS/rGO/Ag⁺-DNA, VitC-derived H₂O₂ reduces the internalized Ag⁺ in AS1411-CS/rGO/Ag⁺-DNA to produce the most silver deposits that instigate the highest ROS oxidative stress (Fig. 3j). This explains why AS1411-CS/rGO/Ag⁺-DNA+VitC attains the highest anti-proliferation efficiency.

Next, the in vivo targeting and anti-tumor activities of AS1411-CS/rGO/Ag⁺-DNA were explored. When mice with HepG2 xenografted tumors were administered with RS-CS/rGO/Ag⁺-DNA via intravenous (i.v.) injection, the fluorescence signal in the tumor was negligible (Fig. 4a). In contrast, in tumor-bearing mice administered with AS1411-CS/rGO/Ag⁺-DNA, the fluorescence signal in the tumor was high for up to 4 h. This can be ascribed to the targeting effect of AS1411, which delivers the SEOB system into the HepG2 tumor cells. Consistent with the in vitro results, high accumulation of the nanosystem favors rGO-enhanced catalytic reduction of Ag⁺ by H₂O₂, which results in abundant silver deposits, and thereby significantly suppresses HepG2 tumor growth (Fig. 4b,c). Inspiringly, the tumors were smallest in the group treated with AS1411-CS/rGO/Ag⁺-DNA+VitC. This result can be ascribed to the fact that VitC enhances the H₂O₂ supply in the tumor cells to facilitate more silver deposits, consequently resulting in the strongest inhibitory effect on HepG2 tumor growth. These results further demonstrate the feasibility of such a SEOB in unlocking the Ag⁺ prodrug and facilitating intratumoral accumulation of silver nanoparticles to suppress tumor growth.

Pathological examination of tumors from AS1411-CS/rGO/Ag⁺-DNA+VitC-treated mice showed some typical characteristics of apoptosis including cell shrinkage, nuclear density increase and nuclear rupture (Fig. 4d). TUNEL immunofluorescence staining revealed that AS1411-CS/rGO/Ag⁺-DNA+VitC achieves the highest level of apoptosis (Fig. 4e). In particular, biological electron microscopic observation of tumor sections indicated the presence of vacuolization and tissue necrosis, which can be regarded as direct evidence to explain the tremendously suppressed tumor growth (Fig. 4f).

General applicability of the SEOB system

The END aptamer has been documented to positively target HepG2 cells due to high expression of the endoglin receptor²⁴. Therefore, we expected that the END-CS/rGO/Ag⁺-DNA system would also inhibit HepG2 tumor growth. END-CS/rGO/Ag⁺-DNA was easily obtained by referring to the synthesis procedure for AS1411-CS/rGO/Ag⁺-DNA (Fig. 2g and Supplementary Fig. S6). The targeting ability of the chelated END aptamer allows more END-CS/rGO/Ag⁺-DNA particles to enter HepG2 cells than RS-CS/rGO/Ag⁺-DNA. Macroscopic silver deposits are produced only in HepG2 cells after adding VitC, which consequently induces massive HepG2 apoptosis (Supplementary Figs S8 and S9). Video S1 clearly shows the astonishing ultra-rapid (15 s) phagocytosis of the green FITC-labeled bioreactor by HepG2 cells due to...
END targeting. This was followed by silver deposit production and apoptosis (represented by red PI staining), due to the potent reduction ability of rGO and VitC-enhanced H$_2$O$_2$. Similar to AS1411-CS/rGO/Ag$^+$-DNA, END-CS/rGO/Ag$^+$-DNA fails to enter other normal cells (e.g., 293T cells) with low endoglin expression, and no evident silver deposits are observed under the same conditions with HepG2 treatment (Supplementary Fig. S8 and Video S2). However, the anti-tumor outcome using END-CS/rGO/Ag$^+$-DNA is inferior to that using AS1411-CS/rGO/Ag$^+$-DNA in the absence or presence of VitC, probably due to differences in their accumulation. This is evidenced by the differences in membrane potential drop, cell viability, DNA damage, ROS production and \textit{in vivo} anti-HepG2 tumor activity (Figs 3 and 4). Despite this, END-CS/rGO/Ag$^+$-DNA remains preferable to other non-targeting groups.

To further demonstrate the general applicability of the AS1411-CS/rGO/Ag$^+$-DNA SEOB prodrug, another xenograft tumor model (i.e., human pulmonary carcinoma A549) was used. The A549 model gave identical results to the HepG2 model in terms of targeting and anti-tumor effects. In detail, AS1411-CS/rGO/Ag$^+$-DNA targets A549 cells much more effectively than RS-CS/rGO/Ag$^+$-DNA, since A549 cells also overexpress nucleolin$^{33}$ (Supplementary Fig. S10). Therefore, the Ag$^+$ pro-drug results in more silver deposits for killing A549 cells, especially in the presence of VitC, and there is no damage to normal L02 liver cells, as evidenced by flow cytometry data (Fig. 5a), trypan blue staining and MTT data (Supplementary Fig. S11). When A549 cells were treated with AS1411-CS/rGO/Ag$^+$-DNA+VitC \textit{in vitro}, strong green JC-1 fluorescence was observed, together with a more evident DNA tailing phenomenon and more ROS production. These results indirectly demonstrate the applicability of the SEOB system to kill tumor cells (Fig. 5b-f). The \textit{in vivo} targeting test reveals that the retention of AS1411-CS/rGO/Ag$^+$-DNA in A549 tumors progressively increases and reaches a peak at 48 h post-injection, while RS-CS/rGO/Ag$^+$-DNA fails to enter tumors (Fig. 5g). This observation validates the excellent targeting ability of AS1411-CS/rGO/Ag$^+$-DNA towards A549. Similar to the results obtained in the anti-HepG2 tumor experiment, the AS1411-CS/rGO/Ag$^+$-DNA prodrug performs best against A549 tumors in the presence of VitC. Tumor inhibition, tumor silver content and tumor cell apoptosis were all elevated when AS1411-CS/rGO/Ag$^+$-DNA was combined with VitC to enhance H$_2$O$_2$ production (Fig. 5h-k).

\textbf{Biosafety evaluation of SEOB}

The biocompatibility of the SEOB-unlocked Ag$^+$ prodrug remains a predominant concern for clinical translation. Herein, normal cynomolgus monkeys were used as a primate model to evaluate the safeties of AS1411- and End-CS/rGO/Ag$^+$-DNA. Astonishingly, histopathological examination of the main organs in cynomolgus monkeys revealed no injuries or apoptosis, which is suggestive of excellent biosafety (Supplementary Fig. S12). This result paves a solid path to clinical translation of the SEOB-unlocked Ag$^+$ prodrug.

\textbf{Discussion}
Based on the high catalytic activity of rGO, we constructed a tumor microenvironment-enabled in situ SEOB to unlock an Ag⁺ prodrug to inhibit various tumors. In this system, intratumoral H₂O₂ acts as the reductant to generate silver nanoparticles and the rGO vehicle acts as the catalyst. The chelated aptamers in the prodrug-carrying bioreactors facilitated targeted uptake of the prodrug by tumor cells. This resulted in production of more silver nanoparticles, especially in combination with VitC, which is capable of supplying massive amounts of H₂O₂ in tumor cells. Consequently, the SEOB system was highly effective against HepG2 and A549 cells in vitro and against HepG2- and A549-derived tumors in vivo. The underlying mechanism is silver nanoparticle-derived apoptosis, associated with ROS-induced mitochondrial membrane potential reduction and DNA damage. The specific aptamer targeting and intratumoral silver nanoparticle production guaranteed excellent biosafety, with no damage to normal cells, since the SEOB system worked only in tumors. More significantly, there was no evident tissue damage in a primate model, which tremendously increases the clinical translation potential of the bioreactors and broadens the application field of intratumoral nanosynthetic medicine.

**Declarations**

**Online methods**

All methods and experimental details are included in the Supplementary Information.

**Data availability**

Additional data related to this paper is available from the corresponding author upon reasonable request.

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**Author contributions**

#Y. Huang and L. Zhong contributed equally to this work. Y. Zhao, Y. Huang and K. Zhang conceived and designed this project. Y. Huang, L. Zhong, Z. Deng, P. Wu, H. Peng, Y. Zhong, L. Liu and J. He performed the experiments. Y. Huang, K. Zhang and Y. Zhao analyzed the data and wrote the manuscript. Y. Zhao and X. Liang supervised the project. Y. Huang, L. Zhong, K. Zhang, X. Liang and Y. Zhao commented on this manuscript.

**Competing interests**
The authors declare no competing financial interests.

Additional information

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