

# Sequential Delivery of Aptamer and STING Agonist Shuttled by Graphene Oxide

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**Abstract.** Stimulator of interferon genes (STING) is a prospective target spot of immunological therapy. The development of STING is still in the preliminary stage and full of challenges. such as lack of tumor specificity, and high-dose-induced T-cell apoptosis. Herein, the STING agonists were selectively delivered and released in the tumor through an aptamer (Apt) modified graphene oxide (GO). The STING agonist was released controllably from GO on account of the acidic tumor microenvironment, and then binds specifically to the STING protein, aiming to active STING pathway and stimulate immunosuppressive factors secretion via phosphorylating TBK1 and IFN IRF3, and secreta high-level IL-6. The resulting immunotherapy exhibited significant inhibition of colorectal tumors, and the producing stimulus was sufficient to activate plentiful enough T-cells with adaptability to inhibit the growth and metastasis of tumors. This work helps to promote sustaining study on the targeted delivery of STING agonists and enhanced activation of the STING pathway, and the translation of novel understanding to clinical applications will enhance the efficiency of immunotherapy for cancer with STING agonists.

**Keywords:** STING agonist; Immunotherapy; graphene oxide (GO); Targeted delivery; MSA-2

## 1. Introduction

Immunotherapy treatments have revolutionized cancer patient care.<sup>[1, 2]</sup> In addition to those immunomodulators directly targeting adaptive immune responses, research on other immunomodulators has been extended to the activation of innate immune, which has the potential to improve tumor immunogenicity.<sup>[3]</sup> Interferon gene stimulating factor (STING) is the receptor inside the endoplasmic reticulum that transmits innate immune perception.<sup>[4]</sup> Activation of the innate immune STING pathway potentiates antitumor immunity is a promising approach to immunotherapy for cancer.<sup>[5]</sup>

Although ideal results have been reported by predecessors, the clinical application of STING agonists still has several great difficulties.<sup>[6, 7]</sup> For example, the expression of STING agonists on vascular endothelium, islet cells, hepatocytes, muscle, epithelium, and mesenchymal stem cells led to STING agonist-based immunotherapy exhibiting "targeted but non-tumor" combined with normal tissues. As a result, systemic injection of STING agonists reduces curative effects and causes serious immune-related undesirable events.<sup>[8, 9]</sup> Several strategies have been developed to integrate agonists into nano-drug carriers, such as liposomes, nanoparticles, hydrogels, etc., to deliver to the whole body or local tissues to avoid non-specific combinations between agonists and normal tissues.<sup>[10-12]</sup> While the reported drug delivery strategies have significant advantages in the treatment of primary tumors, they still face enormous challenges in deep or metastatic tumor therapy.<sup>[13-15]</sup> Moreover, realizing accurate and effective STING activation at the expected target and time also remains challenging.<sup>[16]</sup>

In this study, taking advantage of the immunotherapy, the STING agonist and aptamer were grafted onto the GO (Apt/MSA2@GO), and STING agonist were targeting delivered to tumor cells under the action of aptamer. Due to acidic TME, the STING agonist would dissociate from

Apt/MSA2@GO, and then binds specifically to the STING protein to provoke immunosuppressive factors secretion by phosphorylating tank-combining kinase 1 (TBK1) and IFN regulatory factor 3 (IRF3). Thus, antigen-presenting cells (APCs) are accelerated to mature to promote the proliferation of effector T-cell and enhance anti-tumor immunity.

## 2. Experimental section

**Preparation of Apt/MSA2@GO.** 1 mg of GO was added in 10 mL H<sub>2</sub>O solution included 0.5 mg EDC and 0.7 mg NHS under stirring at 600 rpm/min for 4 h. 0.1 mg aptamer was added in above mixed solution and stirred overnight. The dispersion was then centrifugally washed several times to remove the excess raw materials.

**Characterization of Apt/MSA2@GO.** TEM (JEOL, JEM-2100F, 200 kV) was employed to characterize the morphology of Apt/MSA2@GO. The elemental composition of BPQDs was analyzed by EDS equipped on the TEM instrument.

**Animals and tumor models.** Female BABL/c mice (4–6 weeks) were bought from Zhuhai BesTest biotechnology Co., Ltd. The model of hypodermic colorectal tumor was built by injecting MC38 cells with the quantity of  $1.0 \times 10^6$  into the right side of female BABL/c mice aged 6-8 weeks by 100  $\mu$ L PBS. When tumor sizes reached  $\sim 60$  mm<sup>3</sup>, mice were treated with PBS (control), Apt/MSA2@GO. The number of cytokines in the supernatant was tested using mouse IL-6, STING enzyme-linked immunosorbent assay (ELISA) kits (Multi Sciences) according to the manufacturer's instructions.

## 3. Results and discussion

The schematic representation of Apt/MSA2@GO structure was showed in Fig. 1a. Graphene oxide has a sheet-like structure. The graphene sheet structure remains after loading Aptamer and MSA-2 (Fig. 1b). The results of energy dispersive spectrometer (EDS) element mapping showed that the C, O, P, S atoms were detected (Fig. 1c-1f), which implied that the Apt/MSA2@GO was successfully fabricated.

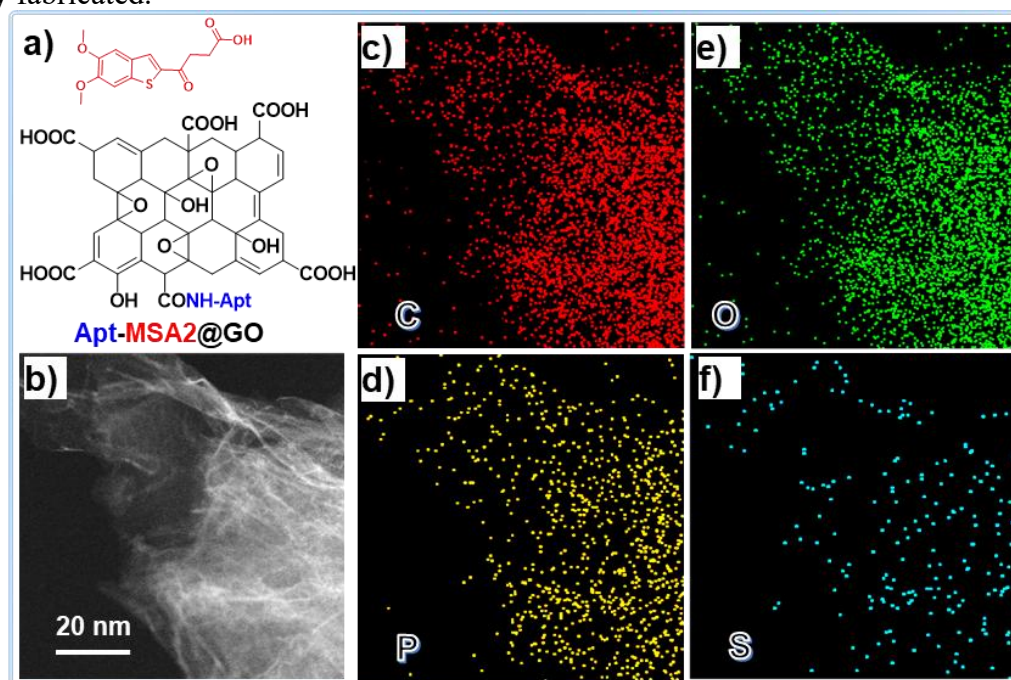


Fig. 1 a) The schematic representation of Apt/MSA2@GO structure; b) TEM images; c-f) EDS element mapping;

The cytotoxicity of different nanocomplexes *in vitro* was investigated by MC38 cell by methyl thiazolyl tetrazolium (MTT) methods as shown in Fig. 2a. At low and high concentrations, no significant cell death occurred was involved, which proved that the prepared materials was safe.

We also conducted animal experiments to test the possibility of the Apt/MSA2@GO for *in vivo* application. The mice were divided into 2 groups in random: i) PBS injection (Control); ii) Apt/MSA2@GO. As shown in Fig. 2b, the colorectal tumor grew fast without any treatment (control group). The tumor volume of the control group reached  $\sim 1800 \text{ mm}^3$ . The targeting immunotherapy of Apt/MSA2@GO showed a valid inhibiting effect to the development of primary tumors. As shown in Fig. 2b, the Apt/MSA2@GO exhibited an obviously slow growth speed of tumor volume, even no measurable tumors appeared when the study ended. The inhibitory rate is up to 100% on the 6<sup>th</sup> day.

Moreover, the primary tumors in group 2 mice all disappeared after being treated and did not return by the 18th day as shown in Fig. 2b, indicating that sustained antigenic stimulation was sufficient to initiate an adequate adaptive T-cell response.<sup>[17, 18]</sup> Furthermore, as shown in Fig. 2c, no obvious inflammation, lesions or histological variations were detected in the heart, liver, lung and kidney of mice in each group, indicating that the treatments exhibit low toxicity to the organs of mice.

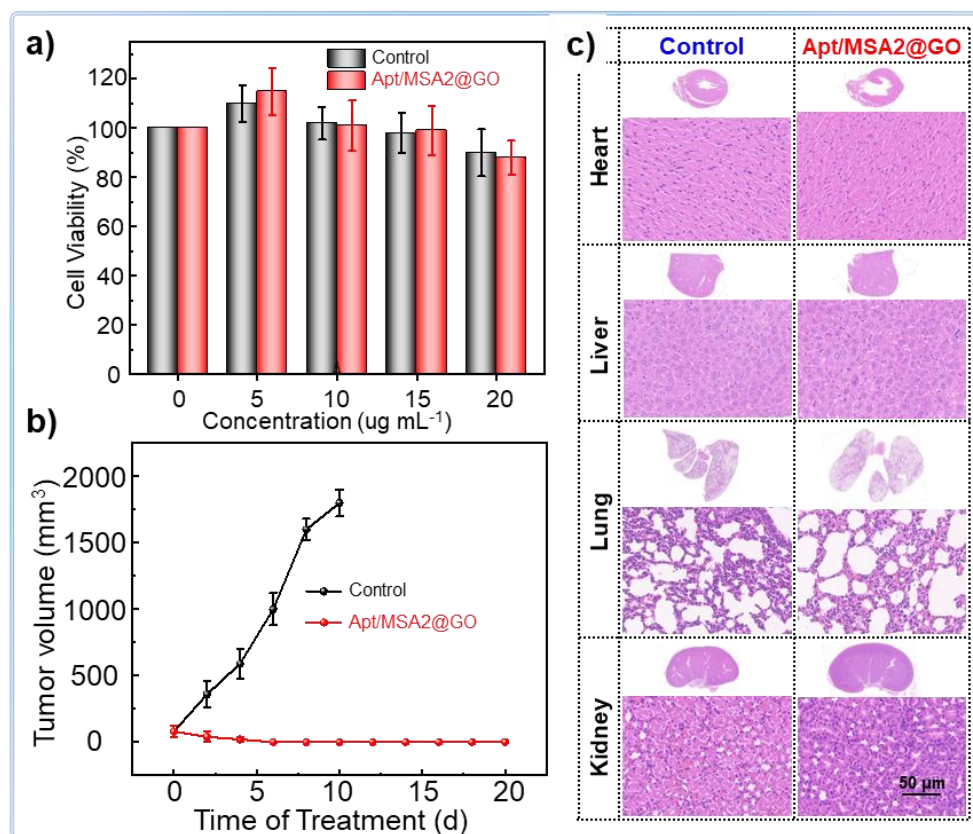


Fig. 2 a) MC38 cell viability of 24 h after different treatments by MTT assay; b) Tumors volume in different groups of mice; c) H&E staining of heart, liver, lung, kidney and tumor after various treatments.

We used ELISA analysis to analyze the fever-related pyrogenic cytokine IL-6 in serum of different treated mice to determine the potential mechanism of Apt/MSA2@GO in inhibiting tumor. As shown in Fig. 3, mice in Group 1 exhibited extremely low level of IL-6, suggesting malignant MC38 colorectal tumor tumors were immunologically “cold” tumors with scarce T-cell infiltration in TME.<sup>[19]</sup> Mice whose tumors were treated with Apt/MSA2@GO show higher levels of IL-6 than the control group. Pro-inflammatory cytokines, acting as the “main adjuster” of the immune system, can heat up tumors.<sup>[20]</sup> The level of STING of mice treated with Apt/MSA2@GO was higher than

that in the free STING agonist, suggesting Apt/MSA2@GO effectively excites the pathway of STING.

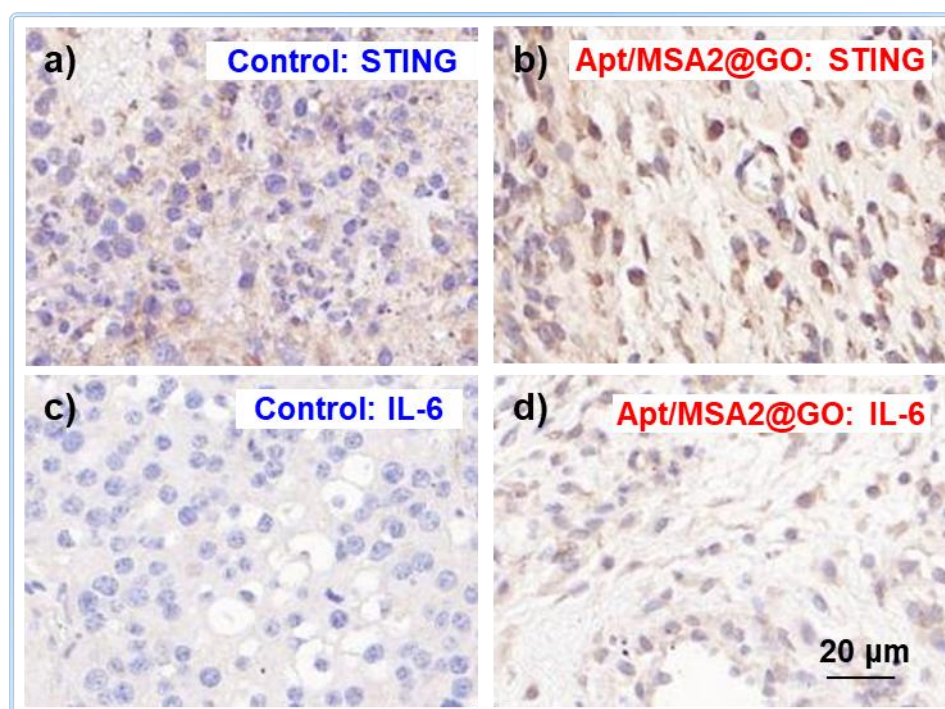


Fig. 3 Immunohistochemistry imaging of Apt/MSA2@GO in MC38 tumor.

#### 4. Summary

In conclusion, we have developed the Apt/MSA2@GO and STING agonists were targeted, and delivered and released in tumors. *In vivo* results show that the immunotherapeutic activity could be specifically enhanced for immunotherapy of colorectal tumor and the tumor was completely eliminated without recurrence and with high safety. The immunotherapy triggered the higher antitumor immune responses, provoking the release of proinflammatory cytokine, and improving the maturation speed of DC, causing the most effective T-cell response to inhibit the growth and metastasis of tumors. The valid antitumor immunity to restrain tumor growth was elicited, and generated a perennial adaptive immune response with adaptability to avoid the relapse of tumor due to the mighty memory of systemic immunity. Moreover, this targeted drug system should be applicable to deliver other small-molecule immune drugs, and the methods of enhancing immunotherapy open up new perspectives in the design of multifunctional nanomedicine platforms.

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