

Novel biodegradable HSAM nanoparticle for drug delivery

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Abstract. The systemic pharmacological treatment of disease is limited by severe toxicity to normal organs/tissue. Therefore, various delivery vehicles have been designed to carry therapeutic drugs to their target tissues. We designed a novel vehicle formed by the interaction of biotins in a DNA (polymer) with avidins (crosslink), resulting in a porous particle. This self-assembled (HSAM) nanoparticle vehicle has been tested in our laboratory both *in vitro* and *in vivo* for its ability to carry doxorubicin, a widely used anticancer drug with a high toxicity to normal organs. Doxorubicin binds to the nanoparticle by intercalating into the DNA strands that are later degraded by nucleases released from cancer cells. Our results showed that 1.1 μg of HSAM DNA can carry 1 μg of doxorubicin, and the doxorubicin-bound HSAM nanoparticle can still be degraded by nucleases (BAL-31 and DNase I). The HSAM nanoparticle carrying doxorubicin can efficiently inhibit cancer cell growth *in vitro* and in a murine model. Furthermore, this nanoparticle is able to deliver up to 180 ng/mg of doxorubicin to the target tumor tissue, which is 15-fold above the systemic toxicity dose (12 mg/kg). These results suggest that the HSAM nanoparticle is both biocompatible and biodegradable, making it a valuable vehicle for drug delivery in cancer treatment.

Introduction

The continuous development of new drug delivery systems is driven by the need to maximize therapeutic activity while minimizing negative side effects. Anticancer drugs, such as doxorubicin and carboplatin, are widely used in cancer chemotherapy. Besides their low water solubility, the major drawbacks of these drugs are the acute toxicity to normal tissue and inherent multi-drug resistance effect. To reduce the acute toxicity of the free drugs and improve their therapeutic efficacy, various delivery vehicles or systems

have been designed, including the liposome (1-3), polymeric micelle systems (2,4) and dendrimers (5).

Although DNA has been used as a therapeutic agent in gene replacement (gene therapy), antisense, siRNA and ribozyme, it has not been used as a drug delivery system to carry toxic drugs to the target organs. A DNA complex can serve as an excellent drug delivery system with several significant advantages: 1) it is a natural component of the human body and, therefore, less harmful to the body and organs (i.e. excellent biocompatibility); 2) it can be efficiently degraded in the body by various nucleases and, therefore, minimize retention problems, i.e. excellent biodegradability; 3) because of its biodegradability, release of the bound drug can be achieved in the target organ; 4) many drugs, such as doxorubicin, naturally bind to DNA as part of their biological mechanisms and would, therefore, bind to the DNA complex without further modification; 5) the binding capacity of the DNA complex can be significantly large; and 6) it is easy to design, manipulate and manufacture. The goal of this study is to engineer a DNA complex using biotinylated DNA and avidin to form a porous DNA structure that can be used as a vehicle to carry an anticancer agent to cancer tissue. In this report, we used doxorubicin, a widely used anticancer drug, as a model system to demonstrate its ability to carry doxorubicin and inhibit squamous cancer cell growth, which is a deadly disease that claims thousands of lives each year.

Materials and methods

Formation of the HSAM nanoparticle. HSAM nanoparticles were formed in 70- μl reactions in the presence of 3×10^{14} molecules (30 μg) of avidin (Sigma-Aldrich, Berlin, Germany) and 4×10^{14} molecules (6.6 μg) of biotinylated HSAM DNA with an avidin to HSAM DNA molar ratio of 3:4. The HSAM DNA was a 31-nucleotide long sequence (5'-TaaccaaagagcaacTacacgaattctcgaT-3') that contained three biotins at positions 1, 16, and 31 (capitalized) (Gene Link, Hawthorne, NY). The mixture of avidin and HSAM DNA was incubated at room temperature for 1 h with shaking at 120 rpm.

Binding doxorubicin to HSAM particles. A doxorubicin solution (30 μl), prepared by the addition of 10 mg of doxorubicin (Sigma) to 2 ml of dimethylsulfoxide and 2 ml of absolute ethanol, was added to 600 μl of HSAM nanoparticle (containing 55 μg of HSAM DNA) as described above. The doxorubicin-HSAM nanoparticle mixture was incubated for 1 h, centrifuged at 3,800 rpm for 3 min, and the

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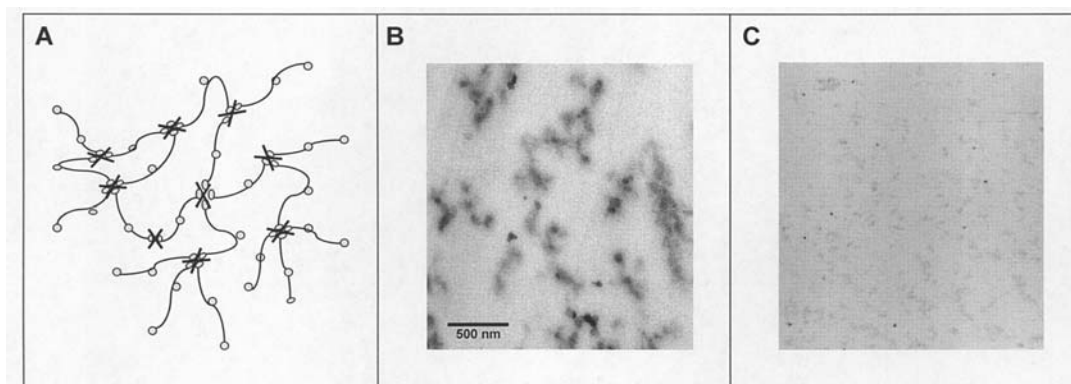


Figure 1. HSAM nanoparticles. (A) Schematic representation of the self-assembled HSAM nanoparticle. Each HSAM nanoparticle is composed of HSAM DNA (curved line) incorporated with three biotins (small circle) and avidins (cross). The HSAM DNA serves as a polymer, and avidin bridges the HSAM DNA together. (B) A representative electronic microscopic image of HSAM nanoparticles. HSAM nanoparticles of various shape and size were identified, ranging from 50-500 nm. (C) A representative light microscopic image of HSAM nanoparticles. HSAM nanoparticles of various shape and size formed within the pores of each particle.

supernatant containing unbound doxorubicin was removed. The doxorubicin-bound HSAM nanoparticle pellet was resuspended in 20 μ l of ddH₂O to a final concentration of 2.75 μ g/ μ l of HSAM DNA, which contained a total of 50 μ g doxorubicin. The mixture was stored at 4°C for later use.

In vitro degradation of HSAM nanoparticles. Enzymatic digestion of the doxorubicin-HSAM nanoparticles was performed in a 15- μ l reaction containing 400 ng of HSAM DNA (with or without doxorubicin) and nuclease BAL-31 (1 U; New England Biolabs, Beverly, MA), mung bean nuclease (10 U, New England Biolabs), exonuclease I (20 U; New England Biolabs), or exonuclease III (100 U; New England Biolabs) in their associated buffers provided by the manufacturers. For DNase I, the reaction was carried out in a 15- μ l reaction containing 40 U DNase I (Gibco BRL, Grand Island, NY), 15 mM Tris-Cl (8.0), 50 mM KCl, and 5 mM MgCl₂. For proteinase K, the reactions were carried out in ddH₂O in the presence of 20 μ g proteinase K, (Roche, Indianapolis, IN). All reactions were incubated at 37°C for 8 h. At the end of incubation, an aliquot of reaction was transferred to a glass slide, and the HSAM nanoparticle was observed under a light or fluorescent microscope.

MTT assay. The percentage of growth inhibition was determined by employing an MTT (3, 4, 5-dimethylthiazol-2, 5-diphenyltetrazolium bromide; Sigma) assay to measure viable cells. A total of 5x10³ cells/well were seeded onto a 96-well plate for 24 h, treated with various amounts of doxorubicin, HSAM nanoparticles, and doxorubicin-HSAM nanoparticles, then incubated for an additional 1-5 days at 37°C. Subsequently, 10 μ l of MTT at a concentration of 4 mg/ml was added to each well, and cells were incubated for an additional 4-6 h. The supernatant was aspirated, and 100 μ l of DMSO was added to the wells to dissolve any precipitate present. The optical density was then measured at a wavelength of 570 nm using an ELx800 reader (Bio-Tek Instruments, Inc.).

Animals studies. Six week-old nude mice (NCR/NU) purchased from Taconic (Cincinnati, OH) were subcutaneously injected

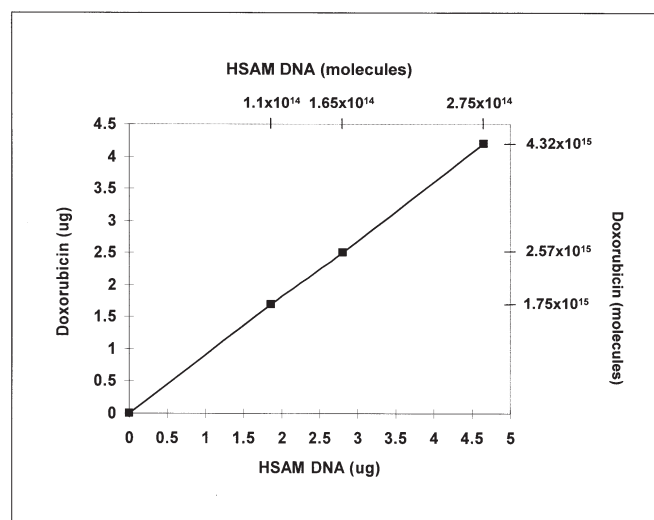


Figure 2. The binding capacity of doxorubicin. Doxorubicin (7.5 μ g) was added to 50 μ l H₂O or HSAM nanoparticle containing various amounts of HSAM DNA (1.86, 2.8 and 4.7 μ g). The absorption of supernatants at OD₂₆₀ was determined using UV spectrophotometry. The amount of doxorubicin bound to HSAM nanoparticles was determined by a decrease in OD₂₆₀ readings, which were taken from the HSAM nanoparticle compared to doxorubicin solution. The results showed that 1.1 μ g of HSAM DNA was able to carry up to 1 μ g of doxorubicin.

with KB cells (5x10⁶) in the right flank. Once tumors reached approximately 100 mm³ (2 weeks), mice were randomized into two experimental groups (n=5), which included a HSAM group (control) and a doxorubicin-HSAM group (treated). In the HSAM group, 20 μ l of HSAM nanoparticles (55 μ g of HSAM DNA) were directly injected into the tumor mass twice a week for 4 weeks. In the doxorubicin-HSAM group, 20 μ l of doxorubicin-HSAM nanoparticles (55 μ g of HSAM DNA) carrying 50 μ g of doxorubicin was injected into the tumor mass twice a week for 4 weeks. Tumor volume and weight were assessed at the beginning of each injection. Tumor volume was measured by two perpendicular dimensions (long and short) using a caliper and calculated using the formula ($a \times b^2$)/2, where a is the larger and b is the smaller dimension of the tumor.

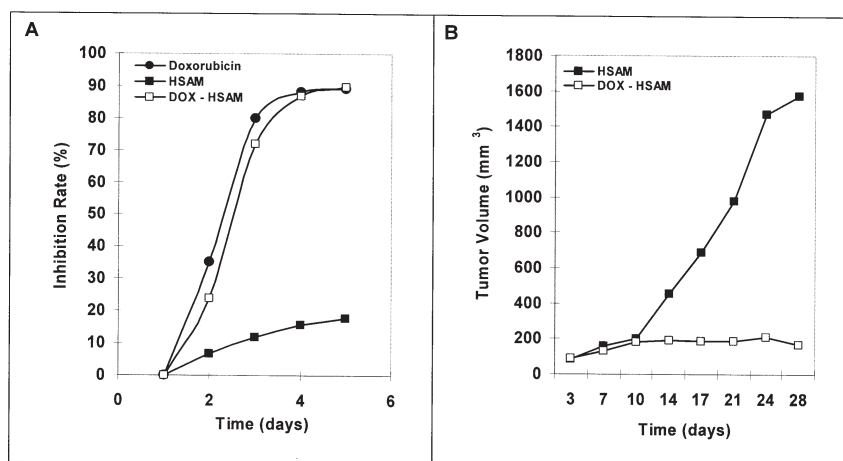


Figure 3. Inhibition of human squamous cancer cell growth. (A) *In vitro* inhibition of KB cell growth. KB cells were seeded onto a 96-well plate at 5×10^3 cells/well and treated with $0.125 \mu\text{g/ml}$ of doxorubicin or $0.138 \mu\text{g/ml}$ of doxorubicin-HSAM containing $0.125 \mu\text{g/ml}$ of doxorubicin. The percentage of growth inhibition was determined by MTT assay on days 1-5. Each experiment was performed in triplicate. (B) Inhibition of tumor growth in nude mice bearing KB cells. Two weeks after subcutaneous inoculation of KB cells, the mice were randomly divided into two groups, 5 in each group. In the control group, $20 \mu\text{l}$ of HSAM nanoparticles ($55 \mu\text{g}$ of HSAM DNA) were injected directly into the tumor. In the treatment group, $20 \mu\text{l}$ of doxorubicin-HSAM nanoparticle ($55 \mu\text{g}$ of HSAM DNA) carrying $50 \mu\text{g}$ of doxorubicin was directly injected into the tumor twice a week for 4 weeks. A significant reduction of tumor volume was observed in the treatment group after 2 weeks of treatment.

Results and discussion

Self-assembled (HSAM) nanoparticles are composed of two types of molecules, biotinylated single-stranded DNA (HSAM DNA) containing three biotins (two at each end and one central) and an avidin. The trivalent HSAM DNA are brought together by the binding of biotins to tetravalent avidins, forming a three dimensional porous structure/particle (Fig. 1A). These self-assembled particles form instantly upon mixing HSAM DNA with avidin due to the high affinity of biotin to avidin ($K_a = 10^{15} \text{ M}^{-1}$) (6). Particle size can increase or decrease when the ratio of avidin to HSAM DNA is varied. For example, at a 3:4 ratio of avidin:HSAM DNA, particles as large as $10 \mu\text{m}$ in diameter (microparticle) are formed. At a 1.5:4 ratio of avidin:HSAM DNA, the diameter of the HSAM particles is $<50 \text{ nm}$ (nanoparticle) (Fig. 1B). At a ratio of 0.3:4, no particles are observed under a light or fluorescent microscope. This phenomenon is similar to the interaction between antigens (multivalent) and antibodies (bivalent) where maximal agglutination occurs when antibody concentration is high enough to approach saturation of half the binding sites of the antigen, and the resulting additional increase of antibody concentration leads to less agglutination (i.e. Heidelberger-Kendall bell-shaped relation) (7).

The ability of the HSAM nanoparticle to carry chemotherapeutic agents was tested using doxorubicin, a widely used and potent anticancer agent. Doxorubicin's mechanism of action interferes with topoisomerase II activity by intercalating into double-stranded DNA (dsDNA) between two GC base pairs (8). However, systemic toxicity, such as cardiotoxicity, mucositis and myelosuppression, limits its clinical use and administered dose (9,10). We hypothesized that doxorubicin can bind to HSAM DNA (thus, HSAM nanoparticles), which can be used as a vehicle to carry doxorubicin to tumor tissue, thereby reducing its systemic toxicity. To determine whether doxorubicin can bind to the

HSAM nanoparticle, $2.5 \mu\text{g}$ of doxorubicin was added to $50 \mu\text{l}$ of HSAM nanoparticle ($4.65 \mu\text{g}$ of HSAM DNA) and briefly incubated at room temperature. Under a light or fluorescent microscope, colorless HSAM nanoparticles became orange-red because of doxorubicin's ability to absorb red light (Fig. 1C). These results indicated that doxorubicin could bind to HSAM DNA. It is interesting to note that binding of doxorubicin to the HSAM nanoparticle made the HSAM nanoparticle tighter, indicating that doxorubicin may bind to different strands of HSAM DNA and bring them closer.

To further determine the binding capacity, we measured the optical density (OD_{260}) value of the supernatant of the mixture of HSAM nanoparticle and doxorubicin and compared it to that of doxorubicin alone. Our results demonstrated that the amount of doxorubicin binding to the HSAM nanoparticle increased with the increase of HSAM nanoparticles (Fig. 2) and the maximal binding capacity was $1 \mu\text{g}$ doxorubicin/ $1.1 \mu\text{g}$ HSAM DNA. These results indicated that approximately 15 molecules of doxorubicin can bind to each HSAM DNA or every two nucleotides can bind to one doxorubicin, which is higher than the previous prediction that every three nucleotides can bind only one doxorubicin (8,11). Our results demonstrated that, although doxorubicin prefers to intercalate into dsDNA (8), it can also bind to single-stranded HSAM DNA (ssDNA). One possible mechanism is that doxorubicin brings together two strands of HSAM DNA, which is consistent with the observation that doxorubicin causes the HSAM nanoparticle to become more compact. We further tested the binding of stable doxorubicin to HSAM DNA. Doxorubicin-HSAM nanoparticles were incubated in ddH_2O for 3 days at room temperature, and the OD_{260} value of the supernatant was determined. Our results showed no significant increase in OD_{260} value, indicating that doxorubicin irreversibly binds to HSAM DNA.

In order for HSAM nanoparticles to carry doxorubicin as a therapeutic agent, doxorubicin has to be released from the

HSAM DNA. Possible biological releasing agents could be proteases that break down avidin and nucleases that break down HSAM DNA. The doxorubicin-HSAM nanoparticles were incubated with proteinase K (a broad-spectrum endopeptidase), DNase I (an endonuclease that digests ssDNA and dsDNA), exonuclease I (digests ssDNA from the 3' end), exonuclease III (digests dsDNA from the 3' end), mung bean nuclease (an exonuclease that digests the ssDNA region of dsDNA from both the 3' and 5' end) and nuclease BAL-31 (single-stranded specific endodeoxyribonuclease with 3' and 5' exonuclease activity). Under a light or fluorescent microscope, both the HSAM and doxorubicin-HSAM nanoparticles decreased after incubation with nuclease BAL-31 and DNase I with similar efficiencies. These results indicated that nucleases gained access to HSAM DNA, and doxorubicin did not interfere with the digestion of HSAM DNA. However, no distinct change in size and/or number of doxorubicin-HSAM nanoparticles was noticed after incubation with other exonucleases. These results indicated that both ends of the HSAM DNA were unavailable for exonuclease activity, probably due to the presence of biotin moieties. In addition, no obvious changes were observed after incubation with proteinase K, which is perhaps due to the inaccessibility of proteinase K to HSAM nanoparticles or protection of avidin from degradation by HSAM DNA.

Although HSAM nanoparticles can be degraded by nucleases, doxorubicin released from the HSAM particle may not be biologically active. Therefore, we tested the ability of doxorubicin-HSAM nanoparticles to inhibit cancer cell growth *in vitro*. The hypothesis was that cancer cells could engulf HSAM nanoparticles and intracellularly degrade them via nucleases. Alternatively, necrotic cancer cells release a large amount of nucleases into pericellular space, which may also degrade HSAM nanoparticles. The *in vitro* viability of KB cells (a human oral squamous cancer cell line) was determined by MTT assay in the presence of doxorubicin, doxorubicin-HSAM nanoparticles and HSAM nanoparticles. Our results showed that doxorubicin alone or doxorubicin-HSAM nanoparticles were able to inhibit KB cell growth and reached 90% inhibition on day 5 (Fig. 3A). These results indicate that doxorubicin released from HSAM nanoparticles maintained its anticancer activity. However, a 3-h delay of growth inhibition was observed with doxorubicin-HSAM nanoparticles compared with doxorubicin alone (at an equal concentration of doxorubicin). The delay in cancer cell growth inhibition by doxorubicin-HSAM nanoparticles was probably due to the delayed release of doxorubicin from the HSAM nanoparticles.

To confirm the anticancer activity of doxorubicin-HSAM particles, we compared tumor growth inhibition in nude mice bearing KB cells by direct intratumoral injections of HSAM nanoparticles and doxorubicin-HSAM nanoparticles. Our results showed that doxorubicin-HSAM nanoparticles suppressed tumor growth for up to 8 weeks. The dose required to suppress tumor growth by direct injection of doxorubicin-HSAM nanoparticles into the tumor was much less than that required by systemic administration (i.p. or i.v.) (12). Furthermore, the concentration of doxorubicin in tumor tissue reached much higher levels than that with systemic delivery of free doxorubicin. A previous study demonstrated that a single dose

of doxorubicin injected through the mouse tail vein can only reach 1 ng/mg in tumor tissue (13), while doxorubicin delivered to the tumor by the HSAM nanoparticle was 180 ng/mg, 180-fold higher. These results indicated that HSAM can serve as a matrix to maintain the drug locally and prevent doxorubicin from being carried away by blood flow, thus achieving a higher intratumoral concentration and reducing systemic toxicity. Upon histological examination, no viable cancer cells were observed in any of the tumors treated with doxorubicin-HSAM particles and the tumors were completely replaced with fibrosis, indicating complete suppression of cancer cell growth. Furthermore, HSAM nanoparticles were not identified in tumor tissue, which is indicative of complete degradation. These results signify that doxorubicin-HSAM nanoparticles can be degraded either intracellularly after endocytosis by tumor cells or in the pericellular space. No toxicity was observed in any animal since the dose (2.5 mg/kg) injected into the tumor mass is much less than the dose required to produce systemic toxicity (LD₅₀ for free doxorubicin via i.p. was 12 mg/kg) (14).

HSAM nanoparticles represent a novel drug delivery system. HSAM nanoparticles can be easily assembled by mixing biotinylated DNA and avidin. The size and shape of HSAM nanoparticles can easily be controlled by adjusting the number of biotins in the HSAM DNA and the ratio of HSAM DNA to avidin. The porous structure of the HSAM nanoparticle is highly permeable, allowing both small and large molecules to be easily packed into the particles. The binding of doxorubicin to HSAM nanoparticles is consistent and stable and does not require a complex chemistry as in other polymer-based drug delivery systems (15). In addition to doxorubicin, other intercalating anticancer drugs, such as daunorubicin, aclacinomycin and mitomycin (10), can be delivered by the HSAM nanoparticle. In this study, we demonstrated that direct injection of the doxorubicin-HSAM nanoparticle into the tumor mass can inhibit tumor growth. This approach has a significant clinical implication. Direct percutaneous injection of anticancer agents into the tumor has been used to treat several solid tumors, such as hepatocellular carcinoma, under ultrasound guidance (16,17) or superficial head and neck cancers by needle injection (18). Slower and localized release of anticancer agents can make this approach more effective than current systemic modalities. The rate of drug release can easily be controlled by the level of resistance of the HSAM nanoparticle to nucleases. For example, a phosphorothioate moiety can be introduced into the HSAM DNA that will increase its resistance to nuclease (19). Another advantage of the HSAM nanoparticle is that it can be used for systemic delivery of doxorubicin because of its small size and temporally controlled release. Targeted delivery of doxorubicin-HSAM nanoparticles to tumor tissue after systemic administration can be achieved by attaching a tumor-specific antibody to the HSAM nanoparticle through biotins attached to the antibody and the avidin of the HSAM nanoparticles (15).

In summary, we demonstrated that a novel biocompatible and biodegradable self-assembled nanoparticle can be used as a vehicle to carry a highly toxic anticancer drug to its target tissues. Therefore, a higher local drug concentration and reduction in systemic toxicity is attained.

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