RESEARCH PAPER

Aptamer AS1411-functionalized gold nanoparticle-melittin complex for targeting MCF-7 breast cancer cell line

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ABSTRACT

Objective(s): Several studies reported the apoptotic and lytic activity of melittin (Mel) in different tumor cells. In this study, a novel nano-complex was developed composed of AS1411aptamers, melittin and gold nanoparticle for the treatment of breast cancer cells.

Materials and Methods: Gold nanoparticles (GNP) were synthesized using reduction of tetrachloroauric acid (HAuCl4). Melittin modified with cysteine and AS1411aptamer conjugated to the gold nanoparticle. Gel retardation assay was used to prove the formation of GNP-Mel-AS1411 complex. Physicochemical properties of complex were investigated by Dynamic Light Scattering (DLS). The cytotoxicity of Mel and GNP-Mel-AS1411 complex were evaluated in both MCF-7 (target) and L929 (non-target) cells by the MTT assay.

Results: The average size of GNP and GNP-Mel-AS1411 complex were 20 ± 2.5 and 270.5± 3.2 respectively. The results of MTT assay revealed that this nanocomplex was more cytotoxic in MCF-7 (cell viability = 19% ± 2%) and less cytotoxic in L929 cells (cell viability = 73% ± 1.6%).

Conclusion: The results of this study indicated that the gold nanoparticle-melittin-AS1411 complex had a potential value in cancer cell targeted delivery of melittin.

Keywords: *AS1411aptamer, Gold nanoparticle, Melittin, Nucleolin, Targeted therapy*

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INTRODUCTION

In recent years female breast cancer has outstripped lung cancer as the most commonly diagnosed cancer and the fifth cause of cancer-related death worldwide. The estimated number of female breast cancer new cases amounts to 2.3 million people with a death number of about 685000 in 2020(1). The primary treatment of breast cancer mainly comprises initial surgical intervention followed by various forms of systemic adjuvant therapy including radiotherapy, chemotherapy, hormonal therapy and immunotherapy (e.g. trastuzumab) (2). Despite all progress made in breast cancer therapy, the current therapeutic strategies suffer from low specificity and deleterious side effects such as cardiotoxicity, neurologic toxicity and myelosuppression (3). Nano-drug delivery systems have the potential to be considered as a promising alternative to common therapeutic approaches to decrease drug toxicity and improve tumor targeting and drug bioavailability. Gold nanoparticles (GNPs) are one of the most common metal inorganic nanoparticles that are attractive candidates for drug delivery thank to their unique properties such as ease

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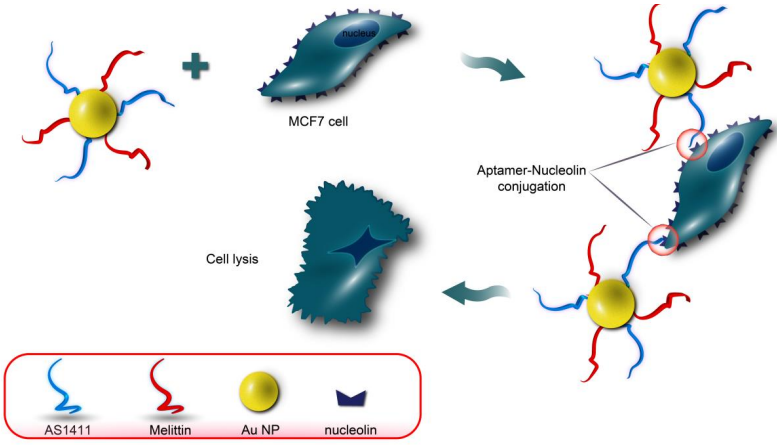

Scheme 1. Schematic description of GNP-Mel -AS1411 complex and its effect on cancer cells

of synthesis, biocompatibility, chemo-physical stability and large ratio of surface to volume (4, 5). Apart from the undeniable advantages of gold nanoparticles, the lack of specificity remains a serious challenge. To overcome this challenge, targeting agents, including aptamers have attracted special attention. Aptamers are short single-stranded oligonucleotides that bind to their target molecules with high affinity and specificity. In contrast to antibodies, aptamers possess such advantages as lower cost of production, more facile synthesis, lower immunogenicity, smaller size, better penetration into target and higher temperature stability. On account of these properties, aptamers have been employed as targeting probes for targeted drug delivery to tumor cells (6). Of them, AS1411, a 26 nucleotide guanine-rich DNA aptamer that adopts a quadruplex structure, specifically recognizes and binds to nucleolin(7). Nucleolin is a protein composed of 707 amino

acids located in the nucleolus of most eukaryotic cells involved in ribosome biogenesis. It has been indicated that nucleolin is overexpressed on the cell surface of several human tumors such as breast, lung, cervical, colorectal, gastric carcinomas, melanoma and glioblastoma(8). Although aptamer AS1411 on its own has been demonstrated to exhibit anti-tumor effects via destabilizing Bcl-2 (9), blocking NF-kB-mediated anti-apoptotic pathways (10) and reducing nucleolin-dependent miRNA levels (11), the present study aimed to augment the toxicity effects of GNPs functionalized with AS1411 via conjugating melittin to GNPs. Melittin (Mel) is a water-soluble cationic peptide of 26 amino acids in length obtained from the bee venom. Because of its positive charge, melittin is able to electrostatically bind to negatively charged cell membranes, resulting in the formation of tetrameric oligomers. The resulting oligomerization leads to pore formation, cellular leakage and finally cell death (12). The highly cytotoxic effects of Mel have encouraged its use to eradicate tumor cells. Despite the potent anti-tumor effects of Mel, its drawbacks, including unsuitable biodistribution, hemolysis, rapid metabolism and off-target toxicity have limited its clinical applications (13). Therefore, in this study a novel AS1411 aptamer-functionalized GNPs were designed as carriers for targeted delivery of Mel to breast tumor cells *in vitro* to minimize the unwanted effects of Mel (scheme 1).

MATERIALS AND METHODS

Reagents

Melittin (Mel) modified with cysteine was custom synthesized and purchased from Shanghai Alkynechem ltd.Tetrachloroauric acid ($HAuCl_4$), (4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT), and TCEP (tris (2-carboxyethyl) phosphine) were purchased from Sigma (USA). AS1411 aptamer, 5’ GGTGGTGGTGGTTGTGGTGGTGGTGG 3’ was obtained from Microsynth (Switzerland). RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were purchased from Gibco (Germany). Ethanol, hydrochloric acid (HCl),dimethylsulfoxide (DMSO) were purchased from MerckCompany (Darmstadt, Germany).

Cell lines

MCF-7 (breast cancer cell) and L929 cell lines were purchased from Pasteur Institute of Tehran, Iran. The cells were cultivated in RPMI 1640supplemented with 10% fetal bovine serum (FBS) and 100 units/ml penicillin-streptomycin at 37 ^{o}C in a humidified atmosphere (95%) containing 5% CO_2.

Synthesis of GNP

Gold nanoparticles were synthesized via reduction of tetrachloroauric acid (HAuCl4) by a modified method of Turkevich (14). In brief, 1 mM $HAuCl_4$ (100 mL) was brought to boil with vigorously stirring. Ten mL of a 38.8 mM sodium citrate solution was added as quickly as possible. After 15 min boiling, the reaction was cooled to room temperature and stored at 4 ˚C until further experiment. The synthesized GNP solution was centrifuged at 10000 g for 20 min at 4 ˚C. After removing supernatant, the pelleted GNPs were resuspended in ultrapure water. The concentration of GNPs was calculated based on extinction coefficient of 2.7 x10^8 M^{-1} cm^{-1} at λ = 520 nm. The zeta potential and the size of prepared GNPs were measured by Zeta sizer (Malvern, UK) (15).

Synthesis of GNP-Mel -AS1411 complex

Cystein-modified Mel was reduced through incubation of 50 µl of modified melittin (5 mM) with the same volume of 50 mM TCEP (tris (2-carboxyethyl) phosphine) as a reducing agent for 1 hr at room temperature prior to initiation of synthesis. Afterwards, reduced Mel along with 50 µl of AS1411 (100 µM) were added to 350 µl of GNPs for 1 hr at room temperature with mild shaking. Further incubation was carried out for overnight at 4 ˚C followed by centrifugation at 10000g for 20 min at 4 ˚C to remove TCEP and the unbound aptamers and Mel. Supernatant was removed and the resulting pellet was resuspended in ultrapure water to final volume of 500 ml (16).

Characterization of GNP-Mel -AS1411 complex

The size and the zeta potential of synthesized complexes were measured by means of a particle size analyzer (Malvern, UK). One milliliter of each compound (GNP-Mel-AS1411, GNP-Mel and GNP-AS1411) were prepared to determine zeta potential and size of the synthesized complexes(17).

Gel electrophoresis

To further confirm formation of desired complex, a 3% agarose gel electrophoresis was performed. The DNA aptamers (10 µM), GNP-AS1411aptamer and GNP-Mel-AS1411 complex, were loaded on the gel. Then, the gel was run at 80 V for 25 min.

MTT assay

The MCF7 and L929 were selected for *in vitro* study of antitumor effects of prepared complex through colorimetric MTT assay.MCF7 categorized as cancer cell lines responding to AS1411 aptamer because it expressed surface nucleolin, facilitating both the binding and uptake of AS1411aptamer (18). L929 consider as nucleolin negative cells as reported by previous studies(19, 20). MCF-7 and L929 cells (5 x10^3 cells per well) were seeded in 96-well plates. After reaching 80% confluency, the cells were exposed to the following compounds: GNP-Mel-AS1411, GNP-Mel, GNP-AS1411, Mel, GNP and AS1411aptamer. The final concentration of Mel and aptamer in each well were 50 and 1 µM, respectively. After 24 hr incubation, medium was removed and replaced with fresh cell culture medium containing 10% MTT solution (stock solution 5 mg/ml) and incubated for 3 hr at 37 ^{o}C. Thereafter, 100 µl of DMSO was added to each well and absorbance was measured by a microplate reader (BioTek, USA) at 490 nm. Each condition was carried out in triplicate (21).

Statistical analysis

The obtained data from MTT assay were statistically analyzed using ANOVA test to compare the means of cell viability percentages between untreated cells and treated ones. All data were presented as means ± standard deviation (SD). In all the tests, *P*-value< 0.05 was considered to be significant statistically.

Table 1. Characteristics of GNP (gold nanoparticles) and functionalized nanocomplex. Each value represents Mean ± standard deviation (n= 3)

Nanoparticles	Z-Average (nm)	Polydispersity index	Zeta potential (mv)
GNP	20 ± 2.5	0.065±0.0001	-27± 1.9
GNP-AS1411	32± 1.9	0.127±0.0035	-44± 2.3
GNP- Mel -AS1411 complex	270.5± 3.2	0.24±0.004	-0.87±0.07

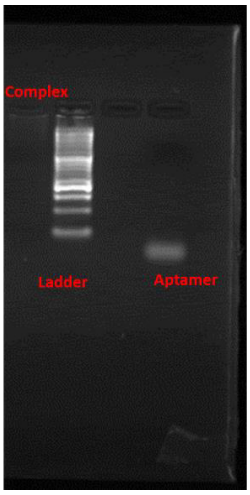

Fig. 1. Agarose gel electrophoresis of from right to left: AS1411 aptamer, empty well, ladder and GNP- Mel-AS1411aptamer (complex)

RESULT AND DISCUSSION

The aim of the present work was to enhance the efficacy of Mel treatment through the application of AS1411 aptamer-conjugated GNP as a targeted carrier.

Fabrication and characterization of the GNP

The citrate-stabilized GNP was synthesized with an average size of 20 ± 2.5 nm and a surface charge of -27± 1.9 mV (Table 1). Subsequently, upon assembly of AS1411aptamer onto the GNP, a change in surface charge from -27± 1.9 mV of single GNP to -44± 2.3 mV was observed implying the successful attachment of aptamer to GNP via van der Waals forces. The average zeta potential value of the GNP increased to -0.87±0.07 after adding Mel, which was attributed to the positive charge of this peptide (22). Consequently, the positive charge facilitated the assembly of the

Table 2. The IC_{50} of different treatments against MCF-7 and L929 cells after 48hrs exposure. Data represented as µM ± standard deviation (n= 3)

Treatment	MCF-7 cells	L929 cells
GNP-AS1411	80.35± 2.7	2500±4.06
GNP-Mel	35.1 ± 1.2	42.37± 1.5
GNP- Mel-AS1411	27.7 ± 3.18	92.5 ± 3.08

GNP-Mel with negatively charged aptamer of AS1411 through electrostatic interaction. Dynamic light scattering of the GNP-AS1411 showed an increase in hydrodynamic diameter from 20 to 32 nm (PDI=0.127). This increase served as the reference for the functionalization of GNP with AS1411 aptamer. DLS measurement showed that the size of DNA-functionalized nanocomplex was about 270.5 ± 3.2 nm (PDI=0.24).

Characterization of prepared complex

Gel retardation assay was used to prove the formation of complex. The band of GNP- Mel -AS1411 complex was retarded relative to the band of AS1411 aptamer, verifying the formation of nanocomplex (Fig. 1).

In vitro antiproliferative activity of GNP- Mel -AS1411 complex

Colorimetric MTT assays were applied to test how GNP- Mel -AS1411 complex affects the proliferation of breast cancer cells (MCF-7) and normal L929 cells as nucleolin positive and nucleolin negative cells, respectively (Fig. 2A and B). At first, Escalating-dose studies of Mel (0–100 µM), showed that modified Mel decreased the cellular viability at higher concentration in comparison to non-modified Mel as we described previously (23). We hypothesized that the lower cytotoxicity potential of modified Mel might be due to adding cysteine residue to amino acid chain

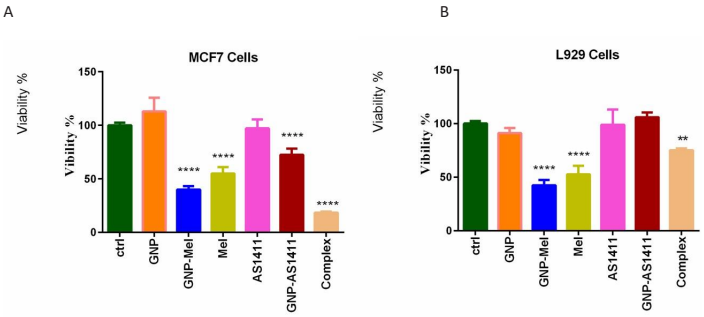

Fig. 2. Graphs showing the effect of GNP, Mel (45 µM for MCF7 and 50 µM for L929), GNP-Mel and GNP-Mel -AS1411 aptamer (final concentration of AS1411 aptamer =1 µM) on the growth of MCF7 and L929 cells after 48 h incubation. Proliferation was measured by MTT assay. Data points represent the mean ± standard error from three independent experiments

of Mel, affecting therapeutic function of protein (24). Although the further studies need to be done so as to confirm the hypothesis. However, the IC_{50} (concentration required to inhibit cell growth by 50%) of Mel for MCF-7 and L929 cells were 45 and 50 µM respectively after 48 hr incubation. We found that neither GNP nor AS1411 aptamer alone was not able to decrease the viability of the cell lines tested, demonstrating its good biocompatibility of GNP as a nanodrug delivery carrier, as was documented in the previous study (25). The impact of prepared complex on cell survival was also tested. The negative charge of AS1411 aptamer in prepared complex seems to prevent the accessibility of complex to membrane of the normal L929 cells (26). So, these cells still maintained higher cell viability after incubation with the GNP- Mel-AS1411 (73 ± 1.6%) compared to GNP-Mel (41± 5%). MCF-7 cell viability after 48 hrs treatments with GNP-Mel and GNP- Mel -AS1411 were 36.42 ± 3.2%, 19 ± 2%, respectively (Table 2). Although GNP-Mel could inhibit MCF-7 cell survival by more than 60%, binding AS1411 aptamer to GNP-Mel led to a remarkable increase in antiproliferative activity of Mel. This is due to the high affinity of AS1411 aptamer for nucleolin molecules present on the plasma membrane of MCF-7 cells. Although a handful of previous studies have applied carriers including perfluorocarbon and PS67-b-PAA 27 polymer to reduce side effect of melittin to normal cells, to the best of our knowledge, this is the first study in which aptamar molecule is employed as probe for targeted delivery of melittin to breast cancer cells along with gold nanoparticle as carrier. In one study an anti-prostate cancer immunoconjugate composed of the first 22 amino acids of melittin, peptide 101 and IgG1 J591 monoclonal antibody (mAb) was used to kill prostate cancer cells. Although the resulting immunoconjugate showed higher toxicity to tumor cells compared to mAb or peptide alone, a high degree of non-specific binding of immunoconjugate was reported that limited its application (27, 28). In contrast, in our study the specificity of aptamer to nucleolin-positive cell line (MCF7) resulted in delivery of toxic agent (melittin) to target cells and rescue of non-target cells (L929) from melittin-induced unwanted cell death. Therefore, aptamer probes promise efficient targeting of tumor cells without significant damage to normal cells. In addition, the use of gold nanoparticles as carrier can ensure the controlled delivery of drug to the site of action and prolong its blood circulation (29, 30). However, the present study revealed that although modification of melittin with cysteine residue decreased its cytotoxic potential, employment of aptamer and gold nanoparticle could drastically increase the specificity of anti-cancer complex.

CONCLUSION

In summary, our study revealed that the conjugation of melittin and AS1411 aptamer on the surface of gold nanoparticle could specifically deliver melittin to MCF-7 cells *in vitro*. This complex could strongly suppressed the breast cancer cells as compared with control, free melittin or gold naoparticle-melittin. Importantly, this complex had less cytotoxic effects in comparison to free melittin or gold naoparticle-melittin on non-target cells.

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CONFLICTS OF INTEREST

No potential conflict of interest was reported by the authors.

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