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Binding and Uptake into Human Hepatocellular Carcinoma Cells of Peptide-Functionalized Gold Nanoparticles

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ABSTRACT: One of the most daunting challenges of nanomedicine is the finding of appropriate targeting agents to deliver suitable payloads precisely to cells affected by malignancies. Even more complex is to achieve the ability to ensure the nanosystems enter those cells. Here we use 2 nm (metal core) gold nanoparticles to target human hepatocellular carcinoma (HepG2) cells stably transfected with the SERPINB3 (SB3) protein. The nanoparticles were coated with a 85:15 mixture of thiols featuring, respectively, a phosphoryl choline, to ensure water solubility and biocompatibility, and a 28-mer peptide corresponding to the amino acid sequence 21-47 of the hepatitis B virus-PreS1 protein (PreS1(21-47)). Conjugation of the peptide was performed via the maleimide-thiol reaction in methanol allowing the use of a limited amount of the targeting molecule. This is an efficient procedure also in the perspective of selecting libraries of new targeting agents. The rationale behind the selection of the peptide is that SB3, which is undetectable in normal hepatocytes, is over-expressed in hepatocellular carcinoma and in hepatoblastoma and has been proposed as a target of the hepatitis B virus (HBV). For the latter the key recognition element is the PreS1(21-47) peptide, which is a fragment of one of the proteins composing the viral envelope. The ability of the conjugated nanoparticles to bind the target protein SB3, expressed in liver cancer cells, was investigated by surface plasmon resonance analysis and *in vitro* via cellular uptake analysis followed by atomic absorption analysis of digested samples. The results showed that the PreS1(21-47) peptide is a suitable targeting agent for cells overexpressing the SB3 protein. Even more important is the evidence that the gold nanoparticles are internalized by the cells. The comparison between the surface plasmon resonance analysis and the cellular uptake studies suggests the presentation of the protein on cell surface is critical for efficient recognition.

INTRODUCTION

Nanomedicine has developed as a platform to allow, in principle, sophisticated and smart drug delivery within the size window of a submicroscopic system that enables delicate and complex interactions with cancer cells and their biological milieu. The size scale of the nanosystems (1-100 nm), closer to proteins and viruses than to molecules, changes the nature of the interactions and,

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3 consequently, distribution in the biological environment with respect to traditional drugs.¹ The
4 self-organized nature of the nanoparticles prepared by bottom-up approaches allows the
5 exploration of new therapeutic and diagnostic modalities^{2,3} also via the exploitation the
6 multivalent and multifunctional properties of the systems.⁴
7

8 Notwithstanding such an amount of advantages, very few nanosystems are currently used
9 in the clinical practice.^{5,6} This is because several issues still need to be addressed for the
10 development of effective nanotheranostic agents, among which targeting is one of the most
11 relevant. First generation nanomedicine agents (including the few liposomal preparations
12 currently approved, which represent the large majority of the nanomedicine agents entered in the
13 clinical use) were based on the enhanced permeation and retention (EPR) effect. Here, the
14 leakiness of the immature tumor vasculature combined with poor lymphatic drainage, causes
15 relatively large (10-100 nm) entities to preferentially accumulate in the cancer tissues.^{7,8} However,
16 EPR effect is not general. Furthermore, nanoparticles larger than the threshold of the renal filters
17 (5 nm) cannot be easily cleared from the organism, leading to long term accumulation.⁹ These
18 problems led to the development of second generation nanosystems based on active targeting
19 strategies, which include conjugation of nanosystems with antibodies or ligands for receptors
20 overexpressed by the target cells.¹⁰ For this reason the selection of new targeting agents is very
21 important and requires a strict collaboration between chemists, biologists, and medical doctors.
22 Furthermore, even when targeting has been achieved, internalization of the nanosystem cannot
23 be taken for granted.¹¹ Several factors affect nanoparticles uptake by cells including charge¹² and
24 the presence of specific peptides (e. g. TAT).¹³ Hence, the synthesis of nanoparticles able not only
25 to target specific cells but also characterized by an enhanced uptake by them would represent a
26 significant achievement.
27

28 In this paper we show how 2-nm diameter monolayer-protected gold nanoparticles
29 conjugated with a 28-mer peptide designed for the targeting of SERPINB3 expressing cells (the
30 PreS1(21-47) fragment) not only bind to the selected target but are internalized into the cells. We
31 do not address their ultimate localization within the cells as this is not a major issue when the aim
32 is cancer cells targeting and, eventually, their killing.
33

34 SERPINB3 (SB3, also known as Squamous Cell Carcinoma Antigen 1, SCCA1) is a soluble
35 serine protease inhibitor of the ovalbumin-serin protease family (ov-serpins). This protein is
36 frequently upregulated in several malignancies of epithelial origin in the liver. Indeed, it is
37 undetectable in normal hepatocytes, but it is over-expressed in hepatocellular carcinoma (HCC)
38 and in hepatoblastoma.^{14,15,16,17,18} Few years ago some of us^{19,20,21} as well as others^{22,23} have
39 demonstrated that SB3 is also a target of the hepatitis B virus (HBV), and a key recognition
40 element is the PreS1(21-47) peptide, which is a fragment of one of the proteins composing the
41 viral envelope. Other target proteins have been suggested for the HBV capsid,²⁴ particularly for the
42 PreS1 region. Our results confirm that SB3 is one of them and the PreS1(21-47) peptide represents
43 not only a new potential targeting agent for hepatic cancer but also for cargo internalization into
44 cells. The implications for possible applications in cancer therapy are obvious.
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RESULTS AND DISCUSSION

In this study, 2-nm metal core gold nanoparticles (AuNPs), coated with a monolayer of thiols were used as candidate nanosystem.^{25,26} The monolayer of organic molecules that protects the nanocluster is made by thiols strongly bound to the gold surface. These thiols typically bear at the opposite end functional groups that tailor the biological properties of the nanoparticles. Preparation of the nanoparticles is much simpler than that of functional polymers or dendrimers, since the single functional units can be synthesized separately and then self-assembled on the gold core. On the other hand, their non-dynamic nature ensures higher stability with respect to liposomes and lipid nanoparticles. For these reasons these nanoparticles represent probably the most suitable platform for the preparation of multivalent functional nanosystems for the ease of preparation, the stability, and the very low toxicity.²⁷

Synthesis and characterization of the AuNPs. We used a two-phase, two-steps synthetic procedure to prepare the monolayer-coated nanoparticles.²⁸ Briefly, the gold cores are prepared in toluene by reduction of HAuCl_4 with NaBH_4 , in the presence of tetrabutylammonium bromide as phase transfer agent and dioctylamine as capping agent. The nanoparticles size is controlled by the amount of dioctylamine, which acts as a weak surface stabilizer. Subsequent addition of a thiol, or of a mixture of thiols, results into the displacement of the amine and the formation of the final protecting monolayer. There are several advantages in the use of this method. First, since the metal core synthesis and thiol monolayer formation are separated, several samples of nanoparticles with the same core size and dispersion and different coatings can be prepared by batch splitting. Second, a good control of the monolayer composition can be achieved. Third, the use of the weaker stabilizing agent (dioctylamine) ensures a smaller size dispersion.

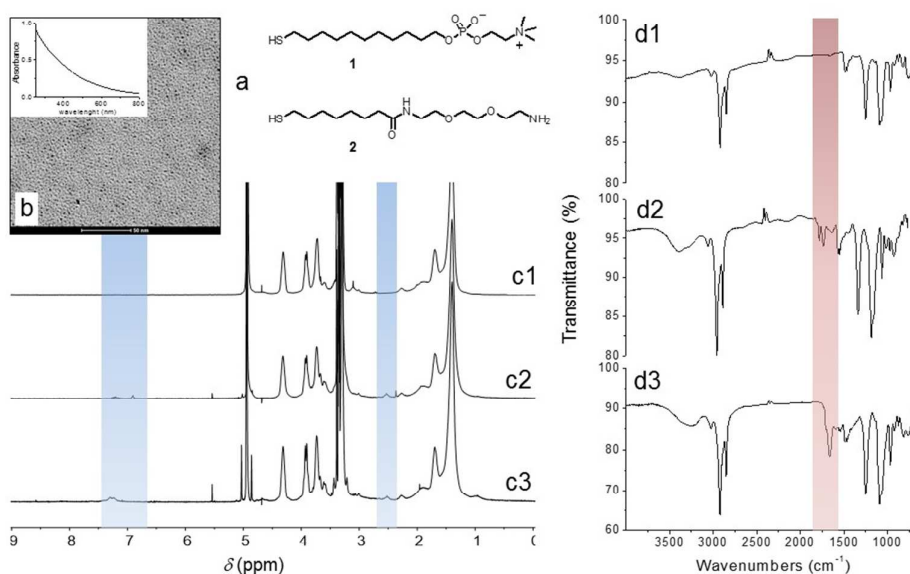


Figure 1. AuNP characterization: a) Thiols used for nanoparticles passivation, b) TEM picture of 1/2-AuNP (inset: UV-Vis spectrum in water); c) $^1\text{H-NMR}$ spectra of 1/2-AuNP (1), 1/2-AuNP after conjugation with MPBS (2), PreS1-AuNPs (3); d) FT-IR spectra of the same samples. The blue and pink strips in the spectra highlight the regions showing the relevant modifications described in the text as a consequence of the conjugation.

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4 Thiols **1** and **2** were selected as coating elements (Figure 1a). Thiol **1**, bearing the
5 zwitterionic and biocompatible phosphorylcholine headgroup, was supposed to give the
6 nanoparticles “stealth” properties by decreasing proteins absorption.^{29,30} Thiol **2** features a
7 terminal primary amino-group easily exploitable for conjugation of the targeting agents. The **1** to **2**
8 ratio in the nanoparticles was adjusted around 10:1 in order to give them targeting properties
9 without affecting multivalency and the ability to escape RES capture. Nanoparticles purification
10 was performed by gel-permeation chromatography. Both **1**-, and **1/2**-coated nanoparticles were
11 found to be fully soluble in water and saline solutions for several weeks. Size of the metal cores
12 from TEM analysis resulted to be 1.8 ± 0.5 nm and 1.9 ± 0.5 nm for **1**-, and **1/2**-coated
13 nanoparticles, respectively (see Figure 1b for **1/2**-AuNP). Such value is confirmed by the UV-Vis
14 spectrum of the nanoparticles, that reveals the absence of the plasmonic absorption band at 520
15 nm (Figure 1b), typical of gold nanoparticles with a metal core diameter larger than 3 nm.
16 Monolayer purity and composition was assessed by ¹H-NMR (see Supporting Information). NMR
17 analysis after iodine decomposition for the **1/2**-coated nanoparticles yielded a **1** to **2** ratio of 92:8.
18 Average formulas for the two nanoparticles is, accordingly, Au₁₈₀**1**₇₀ and Au₁₈₀**1**₆₃**2**₇,
19 respectively.^{31,32}

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21 The CysPreS1(21-47) peptide (CPNFDWDPNNSNAGFAPDLQHDPFFGLP) presents a cysteine
22 at the C terminus of the PreS1(21-47) sequence. The presence of the thiol group in the cysteine
23 side chain allows the easy conjugation to the nanoparticles via maleimide-thiol addition. In
24 principle, one could consider direct conjugation of the peptide to the gold surface. However, this
25 would have resulted in its partial burying in the monolayer constituted by zwitterionic thiol **1** and a
26 loss of peptide surface suitable for binding to the target. The **1/2**-AuNPs were reacted with the
27 crosslinker MPBS in DMF/methanol, to introduce the maleimide functional group, and
28 subsequently conjugated with the CysPreS1(21-47) peptide in methanol to obtain the PreS1-
29 AuNPs. Purification was performed by size exclusion chromatography and effective conjugation
30 was confirmed by NMR and FT-IR spectroscopy (Figures 1c and 1d). Assuming a quantitative
31 conversion of **2** in the peptide conjugate and on the basis of the thiols ratio on the nanoparticles
32 reported above there will be, on average, seven peptides per AuNP.

33
34 In the NMR spectra of **1/2**-coated nanoparticles small signals at 6.5 and 2.3 ppm appeared
35 after MPBS conjugation, arising respectively from the protons of the vinyl group and of the
36 methylene adjacent to the carbonyl group of the crosslinker. Upon reaction with the CysPreS1
37 derivative, the maleimide signal at 6.5 ppm disappeared to be substituted by a broad signal at 7.1
38 ppm, arising from the aromatic side chains of the Phe and Trp residues of PreS1, thus confirming
39 the full conversion of the maleimide group into the conjugate. The FT-IR analysis provided a clear-
40 cut evidence of the presence of the peptide in the purified nanoparticle sample (Figure 1d).
41 Unconjugated particles showed a weak absorption at 1680 nm due to the amide group of thiol **2**.
42 After coupling with MPBS, a new signal at 1720 nm appeared due to the carbonyl groups of the
43 maleimide. Finally, after conjugation with the peptide, absorption at 1680 strongly increased due
44 to the large number of amide groups present in the peptide.

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46 **Interaction of the AuNPs with the target protein SERPINB3.** The ability of the conjugated
47 peptide to interact with the target SB3 protein was first assessed by surface plasmon resonance
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(SPR) analysis, which allows for the direct measurement of the binding events. The SB3 protein was immobilized on a dextrane-coated gold chip via amide coupling (about 2 ng/mm²) and exposed to increasing concentrations of CysPreS1, **1**-AuNPs, and PreS1-AuNPs. Experiments performed with the CysPreS1 peptide did not reveal any interaction (see Supporting Information). Such an outcome was likely the result of the low molecular weight of the peptide, that reduced the amplitude of the instrument response, and of the possible low affinity of the peptide for SB3 at the concentration interval explored.^{20,23} On the other hand, **1**-AuNPs revealed a quite strong binding (Figure 2). Association and dissociation profiles were rather complex and could be fitted only with a kinetic model which includes two different binding modes of the nanoparticles to SB3 (see Supporting Information). Fitting results indicated that both binding events were characterized by very low dissociation constants ($K_{d,1} = 1.1 \times 10^{-11}$ M, $K_{d,2} = 1.8 \times 10^{-11}$ M) but featured different binding and release rates and interfered with each other. The first binding mode was characterized by fast association and dissociation rates ($k_{in} = 8.8 \times 10^9$ M⁻¹sec⁻¹ and $k_{out} = 9.5 \times 10^{-2}$ sec⁻¹) and a low amount of binding sites (8% at low nanoparticles concentrations). The second type of binding sites featured slower binding and dissociation ($k_{in} = 1 \times 10^8$ M⁻¹sec⁻¹ and $k_{out} = 2 \times 10^{-3}$ sec⁻¹). Such sites were predominant (92%) at low nanoparticles concentrations and decreased progressively when the amount of AuNPs injected was increased. Apparently saturation of the first binding sites by increasing the nanoparticle concentration disfavoured the binding to the second. The presence of the PreS1 peptide in PreS1-AuNP led to greater binding (the maximum RU value reached is 200 vs 130 for **1**-AuNP) but did not appear to increase the affinity for the protein ($K_{d,1} = 1.1 \times 10^{-11}$ M, $K_{d,2} = 1.6 \times 10^{-11}$ M). This is probably due to the fact that the high unspecific affinity of the monolayer made by **1** masked the specific binding of PreS1 to SB3 (Figure 2). Control experiments performed using non-functionalized chips or chips functionalized with different proteins confirmed the specific affinity of SB3 for **1**-AuNP (see Supporting Information). This was very disappointing, considering the reported²⁹ little affinity of zwitterionic nanoparticles to proteins. These data do not allow drawing any conclusion on the advantage of functionalizing the nanoparticles with the PreS1 peptide as a targeting agent. We can only state that it does not affect it negatively.

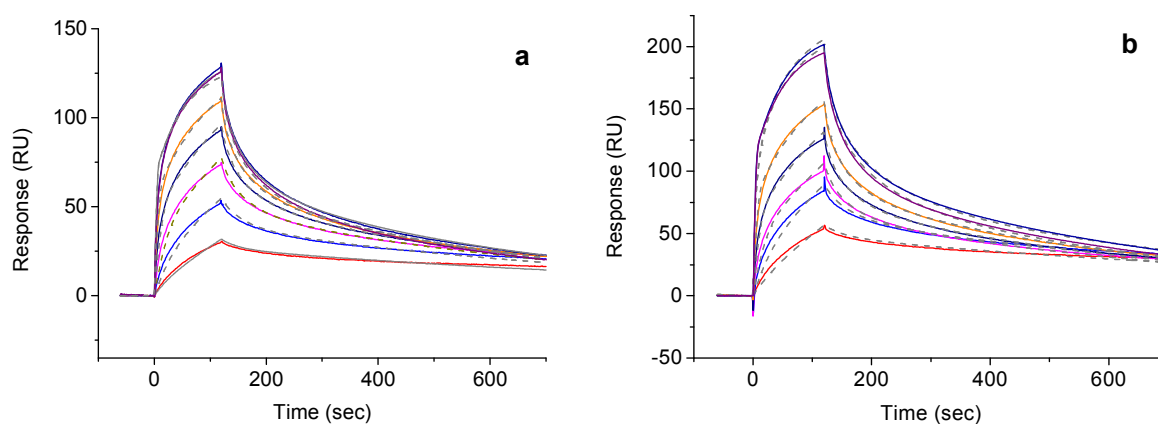


Figure 2. SPR analysis of the interaction between SB3 and nanoparticles: a) Kinetic assays (Multi Cycle) of **1**-AuNP and fitting as heterogeneous ligand; b) Kinetic assays (Multi Cycle) of PreS1-AuNP and fitting as heterogeneous ligand (coloured lines are experimental sensorgrams corresponding to different nanoparticle concentrations, namely red:

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3 0.005 nM, blue: 0.010 nM, magenta: 0.020 nM, black: 0.038 mM, orange: 0.078 nM, dark red and dark blue: 0.157
4 mM; gray dashed lines are the best fitting in both graphs).
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8 A more realistic evaluation of ability of the PreS1-AuNp to selectively bind cancer cells was
9 performed by using human HepG2 cells stably transfected with SB3 (HepG2/SB3 cells). Western
10 blot experiments (see Supporting Information) indicate that, indeed, only HepG2/SB3 cells show a
11 remarkable amount of SB3. These cells were incubated with increasing concentrations of PreS1-
12 AuNPs using HepG2 and **1**-AuNPs as control cells and nanoparticles, respectively. The results
13 (Figure 3) revealed a remarkably higher binding of PreS1-AuNP nanoparticles in the HepG2/SB3
14 cells compared to the controls. In particular, HepG2 cells did not bind either PreS1-AuNPs and **1**-
15 AuNPs nanoparticles confirming the stealth features of the phosphorylcholine coating in the
16 absence of the SB3 protein on the cells. On the other hand, SB3-transfected cells bound both
17 nanoparticles, although at a different extent. In fact, the amount of PreS1-AuNPs bound to
18 HepG2/SB3 cells was substantially higher than that of **1**-AuNPs at any dose and in particular at the
19 lower (0.5 $\mu\text{g}/\text{mL}$) concentration used. On one side, these data indicated an increased uptake of
20 the nanoparticles conjugated with the targeting peptide mediated by its interaction with the SB3
21 protein, on the other they also confirmed the affinity of the phosphorylcholine coating for SB3
22 revealed by the SPR experiments. The better affinity of the PreS1-AuNPs for HepG2/SB3 cells
23 compared to **1**-AuNPs in these experiments indicated a lower unspecific interaction of the
24 zwitterionic component of the monolayer or, more likely, a more appropriate presentation of SB3
25 on the cells surface than on the chips used for the SPR experiments. It is not infrequent the
26 immobilization of a protein on the surface for an SPR experiment alters its interaction with the
27 target.
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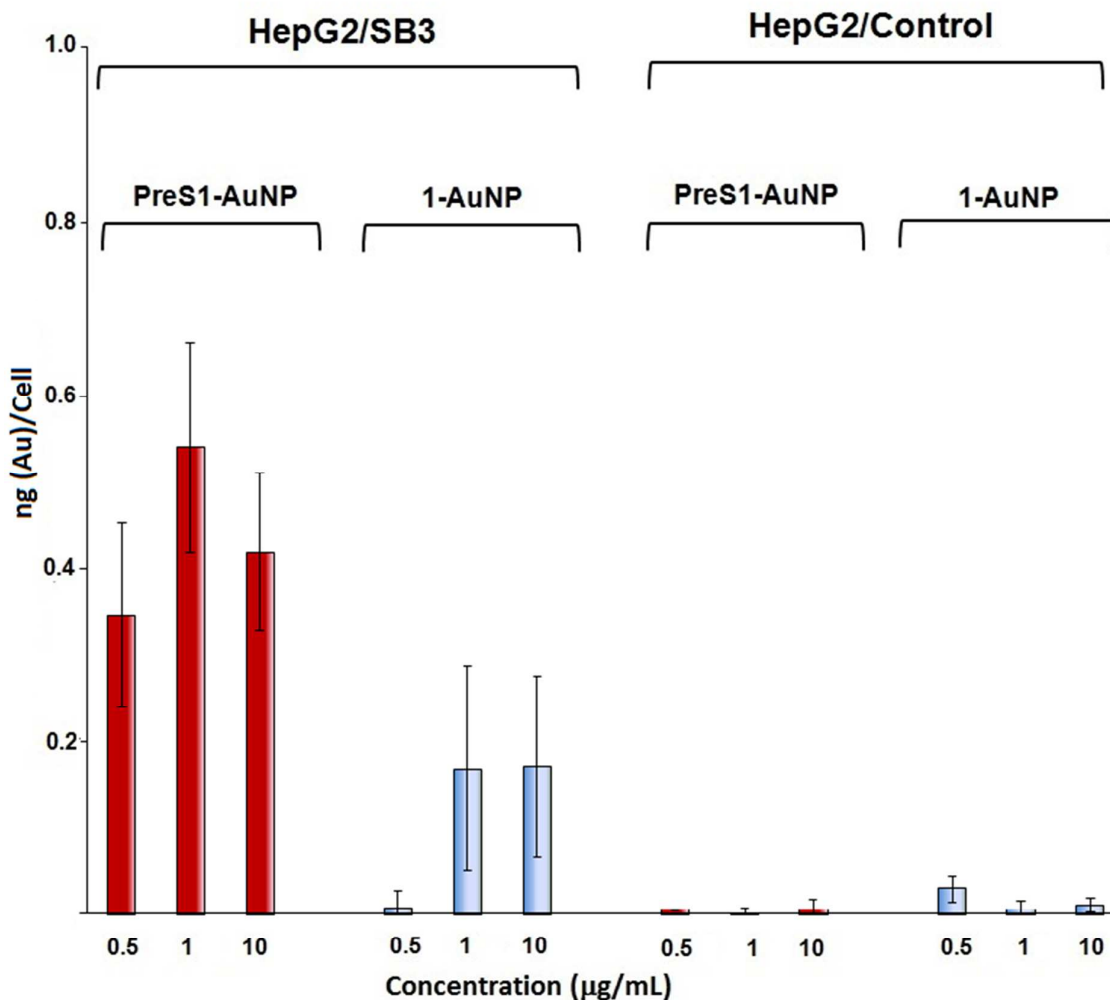


Figure 3 Binding specificity analysis of nanoparticles to HepG2/SB3 cells and controls (HepG2/control). Conditions: 1 hour incubation at 37 °C and 5% CO₂. Bars represent mean values ± SD of two different sets of experiments (two runs each). The gold cellular content was determined by atomic absorption analysis of cellular pellets. The X axis shows the different concentration of AuNPs utilized.

The dose-response trend was quite flat, suggesting a threshold uptake of the nanoparticles is already reached at low concentrations. Consequently, we selected 1 μg/mL as the best experimental concentration at 1 hour incubation to assess the fate of the nanoparticles and, in particular their internalization into the cells. As reported in Figure 4, binding of PreS1-AuNP was more than 6 times higher than that of the nanoparticles without the peptide (1-AuNP). Trypsin treatment of HepG2/SB3 cells, which, as we have as previously reported allows to differentiate, at least in part, between HBV binding and internalization,²¹ did not cause any decrease of the amount of gold bound to the cells. This suggests that the nanoparticles were internalized and not just bound to the cell surface. This adds another possibility to explain the difference observed in the SPR and in vitro experiments. In fact it could be that binding is not much enhanced by SB3 presence on the surface of the cells but whenever it occurs it induces efficient internalization of

the nanoparticles in them. We cannot exclude that this is due, at least in part, to an overall difference in endocytosis activity induced by SerpinB3. In any case the presence of the PreS1 peptide is a requisite for the process to occur.

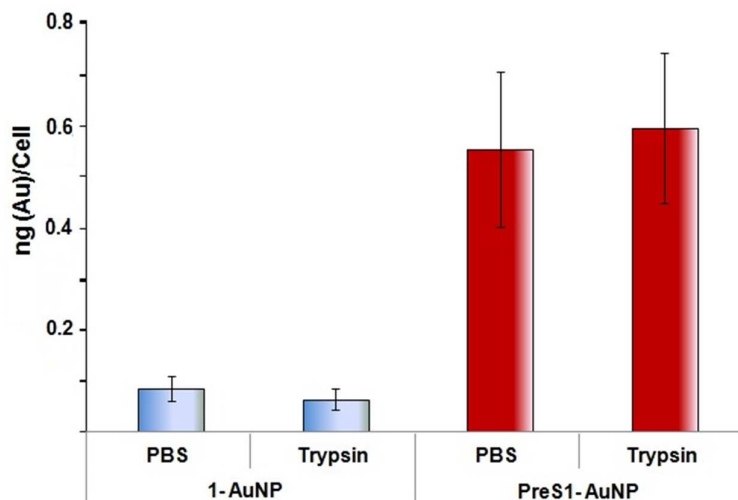


Figure 4. Nanoparticles uptake by HepG2/SB3 cells. Conditions: 1 hour incubation at 1 $\mu\text{g}/\text{mL}$ concentration with or without 5 minutes treatment with trypsin at 37°C. Negative controls were treated with PBS. The gold cellular content was determined by atomic absorption analysis of cellular pellets. Bars represent mean values \pm SD of three different experiments.

Cellular toxicity of the AuNPs was investigated by monitoring the cell growth curves upon incubation with and without the nanoparticles. All the curves were identical over the time interval investigated (96 h) indicating no cytotoxicity induced by the nanoparticles (Figure 5).

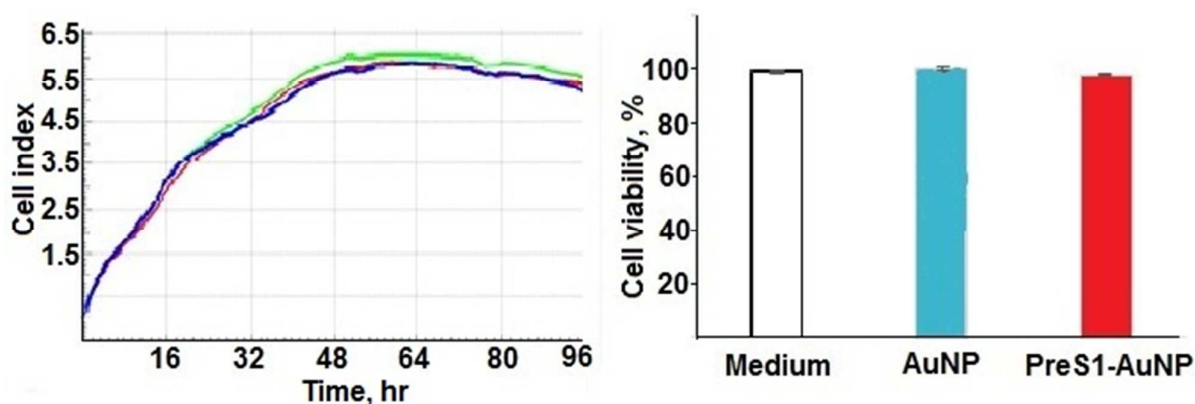


Figure 5. Left panel: time course of the cell growth in the medium (blue trace), in the presence of 1 $\mu\text{g}/\text{mL}$ of 1-AuNP (red) or PreS1-AuNP (green); the cell index is a relative measure of cell number present in a well. Results are expressed as mean of automatically calculated cell indexes of three different experiments. Right panel: cell viability of the same solutions after 24 h.

CONCLUSION

In conclusion, we reported here the facile preparation of stable, water soluble 2-nm gold nanoparticles that can be easily conjugated with a peptide bearing a thiol group.^{33,34,35} With respect to other conjugation methods, the use of the maleimide-thiol reaction in methanol allows the targeting molecule to be fully exposed on the surface of the nanoparticle thus ensuring a better presentation to the target. The data we have obtained put in evidence that the PreS1(21-47) peptide is a suitable targeting agent for cells overexpressing the SB3 protein. Even more important is the evidence that the gold nanoparticles are internalized by the cells. The mechanism of internalization of the virus is far from being understood.^{36,37} It appears that the PreS1-SB3 interaction is not the only factor. For instance, Hao et al.³⁸ have suggested that ferritin light chain (FTL) is involved in the formation of a putative preS-FTL-SB3 triple complex. The SPR results, if we accept the hypothesis of a wrong orientation of SB3 on the chip surface, indicate a precise presentation of the protein to the virus surface is required for recognition. Harrison et al.²² have shown that the binding event does not involve the reactive site loop of SB3. This could imply the binding occurs at the surface of the protein supporting the suggestion that a wrong orientation is the cause of the unselective binding in the SPR experiments. Regardless the precise mechanism of binding and internalization our results are important in view of a possible use of these nanoparticles as carriers to deliver and internalize a suitable payload in hepatocellular carcinoma treatment. Nanoparticle delivery to malignant tissues and cells is an hot topic Efficiency in the process, particularly as tumors are concerned, has been shown recently to be still very challenging.³⁹

EXPERIMENTAL PROCEDURES

Syntheses. Thiols **1** and **2** were prepared following previously published procedures.^{30,40} Peptide PreS1(21-47) was purchased from JTP, Germany. Its purity was checked by HPLC and found to be better than 99%. Conjugation to the nanoparticles and functionalization of the peptide were performed following standard protocols. Details are reported in the Supporting Information.

Nanoparticles characterization. Nanoparticles ¹H-NMR spectra were recorded using a Bruker AV300 spectrometer operating at 300 MHz. About 10 mg of nanoparticle were dissolved in 0.6 ml of CD₃OD and the spectrum was recorded. TEM images were recorded on a Jeol 300 PX electron microscope. One drop of sample solution was placed on the sample grid and the solvent was allowed to evaporate. UV-Visible spectra and kinetic traces were recorded on a Cary 50 spectrophotometer equipped with thermostatted multiple cell holders. IR spectra were recorded on a Nicolet 5700 FT-IR spectrophotometer and reported in cm⁻¹. AuNPs were dissolved in methanol at the concentration of 10 mg/mL then 20 μL of the solution casted onto a NaCl plate by an Eppendorf micropipette and dried using a hotplate. NMR spectra and TEM images are in Fig. 1 and in the Supporting Information.

Surface Plasmon Resonance analysis. SPR analyses were performed using a double channel Biacore X100 instrument (GE Healthcare, Uppsala, Sweden). Recombinant SB3 was produced after cloning human SB3 cDNA in the directional expression vector pET101 (Invitrogen Carlsbad, CA), as previously described.⁴¹ A dextrane-coated gold chip (CM5) was activated by flowing a 1:1 mixture of 0.2 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccinimide in water. A continuous flow of HEPES pH 7.4 was maintained. SB3 (50 μg/mL) in 10 mM sodium acetate (pH 5) was immobilized on the activated chip surface at a flow rate of 10 μL/min to obtain an immobilization level of "Response Bound" of 1661 RU and "Response Final" of 1840 RU. Excess of activated carboxylic groups on the chip was blocked with ethanolamine. Control channel was treated following the same protocol without protein immobilization. Kinetic assays were performed with both PreS1AuNPs and **1**-AuNP as control, following the instrument built-in standard protocol using HBS-EP+ buffer at pH 7.4 (0.1 M HEPES, 1.5 M NaCl, 30 mM EDTA, 0.5% v/v P20 surfactant) by subsequent 180 sec injections of nanoparticle solutions at increasing concentration (0.005-0.157 nM) using NaCl 3 M as regeneration solution. Details are reported in the supporting information.

Cellular uptake and viability analysis. To analyze the ability of nanoparticles to selectively bind SB3 in cells manipulated to over-express this serpin, *in vitro* experiments were carried out. HepG2 cells, derived from a human hepatoma (LGC Standards S.r.l., Sesto San Giovanni, MI, Italy) were stably transfected with a plasmid vector carrying the wild-type SB3 human gene (HepG2/SB3) or with the plasmid vector alone (HepG2/empty vector) (pcDNA3.1D/V5-His-TOPO, Invitrogen Life Technologies, NY, USA) as previously reported.⁴² In preliminary experiments both cell lines were tested for SerpinB3 protein content by Western blot and only HepG2/SB3 showed a remarkable amount of this serpin (Supporting Information Figure S19). Both cell lines were initially incubated for 1 hour with increasing amounts (0.5, 1, 10 μg/ml) of PreS1-AuNPs or **1**-AuNPs, as negative control, to define the best experimental conditions and to test the specificity of

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3 functionalized nanoparticles binding to SB3 target protein. In order to evaluate if PreS1-AuNPs
4 were internalized into SB3-expressing cells, trypsin treatment was carried out for 5' at 37°C after 1
5 hour incubation. Negative controls were treated with PBS for the same time frame. Real-time
6 monitoring of the potential cytotoxicity of nanoparticles was carried out using the xCELLigence DP
7 instrument (Roche Diagnostics, Mannheim, Germany) as described in the supplier's instruction
8 manual. HepG2/SERPINB3 cells were seeded (50,000 cells/well) in the E-Plate 96, maintained at
9 37°C in 5% CO₂ atmosphere and treated with 1 µg/mL of PreS1AuNPs or of 1-AuNPs or with
10 medium. Cell cultures not treated with nanoparticles were used as control. The cell Index values (a
11 quantitative measure of the cell number present in a well) were achieved using the RTC software
12 (version 1.2, Roche Diagnostics) and monitored every 10 minutes for 96 hours.

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17 **Atomic absorption analysis.** In order to analyze the amount of nanoparticles entered into
18 the cells, atomic absorption analysis was carried out. The digestion of cellular pellets was
19 accomplished by addition of 0.5 ml of aqua regia (HCl 36% : HNO₃ 3%=3:1) to each cell sample
20 followed by heating at 70°C for 1 h in a sealed vial. Subsequently, an aliquot of 250 µl of digestion
21 solution was transferred into a 15 ml polypropylene tube and filled to a total volume of 10 ml with
22 a 3% solution of HNO₃. Gold content was determined by standard Atomic Absorption
23 measurements.

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26 **Western blot analysis.** Fifty µg of total protein content from each cellular extract, obtained
27 by cellular protein extraction using RIPA lysis buffer, were loaded on 10% polyacrylamide gel.
28 Antigenic detection was carried out by enhanced chemiluminescence (Amersham, Arlington
29 Height, IL) and densitometric analysis was assessed using VersaDoc Imaging system (Bio-Rad
30 Laboratories, Hercules, CA). The following antibodies were used: monoclonal anti-SERPINB3
31 antibody (0.5 µg/ml, R&D SYSTEMS Minneapolis, MN), monoclonal anti-β-actin antibody (1:1000,
32 Sigma-Aldrich, St. Luis, MO) and anti-mouse horseradish peroxidase conjugated secondary
33 antibody (1:1000, Amersham Bioscience, Arlington Height, IL).

34 35 36 37 38 39 40 ASSOCIATED CONTENT

41 Supporting Information

42 The Supporting Information is available free of charge on the ACS Publications website at DOI:

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53 Notes

54 The authors declare no competing financial interests.

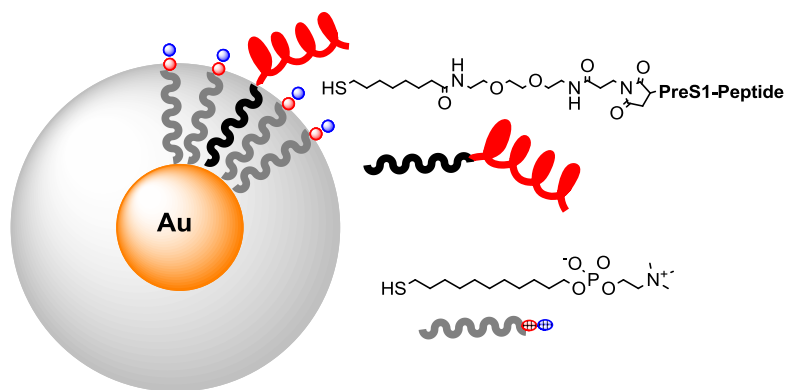
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