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1 **Selective anticancer activity of hydroxyapatite/chitosan-poly(D,L)-lactide-**
2 **co-glycolide particles loaded with an androstane-based cancer inhibitor**

3
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11
12 **Abstract:**

13 In an earlier study we demonstrated that hydroxyapatite nanoparticles coated with chitosan-
14 poly(D,L)-lactide-co-glycolide (HAp/Ch-PLGA) target lungs following their intravenous
15 injection into mice. In this study we utilize an emulsification process and freeze drying to load
16 the composite HAp/Ch-PLGA particles with 17β -hydroxy- 17α -picolyl-androst-5-en- 3β -yl-
17 acetate (A), a chemotherapeutic derivative of androstane and a novel compound with a
18 selective anticancer activity against lung cancer cells. ^1H NMR and ^{13}C NMR techniques
19 confirmed the intact structure of the derivative A following its entrapment within HAp/Ch-
20 PLGA particles. The thermogravimetric and differential thermal analyses coupled with mass
21 spectrometry were used to assess the thermal degradation products and properties of A-loaded
22 HAp/Ch-PLGA. The loading efficiency, as indicated by the comparison of enthalpies of phase
23 transitions in pure A and A-loaded HAp/Ch-PLGA, equaled 7.47 wt.%. The release of A from
24 HAp/Ch-PLGA was sustained, neither exhibiting a burst release nor plateauing after three
25 weeks. Atomic force microscopy and particle size distribution analyses were used to confirm
26 that the particles were spherical with a uniform size distribution of $d_{50} = 168$ nm. *In vitro*
27 cytotoxicity testing of A-loaded HAp/Ch-PLGA using MTT and trypan blue dye exclusion
28 assays demonstrated that the particles were cytotoxic to the A549 human lung carcinoma cell

1 line (46±2%), while simultaneously preserving high viability (83±3%) of regular MRC5
2 human lung fibroblasts and causing no harm to primary mouse lung fibroblasts. In conclusion,
3 composite A-loaded HAp/Ch-PLGA particles could be seen as promising drug delivery
4 platforms for selective cancer therapies, targeting malignant cells for destruction, while
5 having a significantly lesser cytotoxic effect on the healthy cells.

6

7 **Keywords:** *androstane; chitosan; hydroxyapatite; lung cancer; nanoparticle; PLGA.*

8

9 **1. Introduction**

10 The potential for application of synthetic calcium phosphates, especially hydroxyapatite
11 (HAp), has become intensely broadened in the past 15 years. It has extended from bone tissue
12 engineering to multiple other fields of biomedicine, e.g., controlled drug delivery, bioactive
13 coatings, gene therapies, wound healing and beyond [1-3]. The enhancement of the properties
14 of HAp in this pursuit of new areas of application has progressed in two major directions: (a)
15 chemical modification and (b) combination with other materials, predominantly polymers. As
16 far as the first direction is concerned, HAp surpasses other biominerals in terms of the
17 capacity to incorporate foreign ions, some of which can be utilized for therapeutic purposes,
18 substituting the role of costlier biomolecules and minimizing the side effects frequently
19 associated with their use [4]. To that end, tailoring of the chemical structure of HAp has been
20 achieved through the substitution of its constitutive ions (Ca^{2+} , PO_4^{3-} and OH^-) with ions such
21 as Mg^{2+} , Zn^{2+} , Cd^{2+} , Y^{3+} , Mn^{2+} , Ni^{2+} , carbonate, selenite, silicate, etc. [5, 6]. Cobalt-
22 substituted HAp, for example, accelerated the process of osseointegration after the
23 reconstruction of bone defects *in vivo* [7].

24 Progress in the field of composite nanoparticle synthesis has, on the other hand,
25 enabled the fabrication of a large number of hybrid, multicomponent systems containing

1 HAp. Biocompatible polymeric coatings around HAp nanoparticles have allowed for the
2 development of multifunctional nanosystems as carriers of vitamins, antibiotics and genes [8-
3 13]. The tailoring of the structure of multifunctional nanoparticulate drug carriers is an
4 emerging branch of research, with the goal of enhancing their biocompatibility and
5 therapeutic efficacy [9, 14]. Multifunctional systems exhibit a more convoluted behavior in
6 the course of *in vitro* and *in vivo* testing and also demand more versatile techniques for their
7 physicochemical characterization [14]. Nanostructured HAp has been extensively used in the
8 reconstruction of boney tissues and it has also showed promising results as a system with a
9 targeted activity in the treatment of osteosarcoma metastases [15]. HAp nanoparticles can
10 slow down the growth of various cancer cells, particularly human glioma cells [16]. Organ-
11 targeting or tumor-targeting via drug-loaded nanoparticles demonstrated their ability to act on
12 specific receptors [17]. The targeting of receptors by HAp nanocrystals was proposed as the
13 basis for the utilization of HAp doped with rare earth elements for noninvasive medical
14 imaging at the molecular level [18]. Our previous research on nano/micro-particle systems
15 composed of HAp coated with the chitosan-poly(D,L)-lactide-co-glycolide polymer blend
16 (HAp/Ch-PLGA) has justified the use of HAp as a core component of composite drug carriers
17 for organ-targeting therapies [19]. Along with the particle size, shape and composition, the
18 fate of particles injected into the bloodstream is mainly defined by the type and the structure
19 of proteins bound to them [20, 21], which can be controlled by tailoring the composition, the
20 topography and the charge distribution on the particle surface. Coating HAp nanoparticles
21 with different polymers has correspondingly directed HAp to different organs [19].
22 Specifically, unlike pure HAp and HAp coated with PLGA only, which were being
23 accumulated in liver, spleen and kidneys, HAp/Ch-PLGA particles were overwhelmingly
24 localizing in the lungs of mice following the intravenous injection of the particulate dispersion
25 [19]. Bone defect reparations have been successfully performed using nanoparticulate systems

1 based on PLGA-coated HAp, providing a rationale for the consideration of PLGA as a coating
2 for organ-targeting delivery systems [10-12]. One of the advantages of mixing PLGA with
3 chitosan within nanoparticulate platforms for controlled delivery of chemotherapeutics in the
4 treatment of cancer is an improved permeation of the cell membrane [22]. The enhanced
5 sialylation of malignant cells [23] benefits the idea of using cationic Ch to target cancer cells
6 and may be the reason behind its occasionally observed anticancer properties [24]. The
7 previously observed ability of the positively charged molecules and particles to localize in
8 lungs [25-28] may also be a key to explaining the pronounced lung-targeting potential of
9 HAp/Ch-PLGA particles [19].

10 Lung cancer is the leading cause of cancer mortality [29]. Estrogen and progesterone
11 are hormones that play an important role in the carcinogenesis of lung cells [30], placing this
12 type of cancer in the category of hormone-dependent cancers. The 17-picolyl, 17-
13 picolinylidene, 17-picolinylidene-16-one and 17-picolyl-16-one androstane derivatives are
14 effective cell inhibitors of hormone-dependent cancers, e.g., adenocarcinoma, prostate cancer,
15 cervix carcinoma, colon cancer, etc. [31-33]. This explains their choice as a chemotherapeutic
16 drug to be delivered to lung cancer cells in this study. As for the choice of the carrier, HAp
17 nanoparticles have been used with success to target tumors through vasculature and extend
18 life expectancy in animal studies. For example, HAp nanoparticles, albeit doped with selenite
19 ions and injected into the bloodstream, extended the lifetime of mice with hepatocellular
20 carcinoma [34]. Also, a direct injection of HAp nanoparticles into a transplanted tumor
21 formed by Bel-7402 human hepatocarcinoma cells in nude mice led to a 50 % reduction in the
22 tumor size [35]. On the other hand, the antiproliferative activity of HAp was twice more
23 intense for cancer cells than for the normal ones, both in vitro and in vivo [35]. The selective
24 cytotoxicity exhibited by HAp was also not due to the release of reactive oxygen species [35]
25 (as is the case with, e.g., carbon nanotubes, quantum dots, Ag nanoparticles), as this would

1 cause unselective cytotoxicity, targeting both cancerous and healthy cells [36]. Rather,
2 nanosized HAp particles get internalized by cancer cells and localized around the
3 endoplasmic reticulum (ER), where they presumably interfere with the translation process by
4 competing for ribosome binding together with mRNA, thus arresting the cell cycle in G0/G1
5 phase [35]. Interestingly, the androstane derivative chosen as the anticancer drug for this
6 study owes its selective anticancer activity to a similar effect, i.e., the induction of the ER
7 stress response, resulting in calcium release from the ER and the G1 phase cycle arrest [37].
8 Given the similar mechanism by which HAp and the compound A inhibit the proliferation of
9 cancer cells, a possible therapeutic synergy between the carrier (HAp) and the drug (A) is
10 worth investigating.

11 In our previous study we demonstrated the ability of nanoparticles composed of HAp
12 coated with Ch-PLGA polymer blend to target lungs following intravenous administration
13 [19]. In this study we describe the fabrication of androstane (17 β -hydroxy-17 α -picolyl-
14 androst-5-en-3 β -yl acetate, A)-loaded HAp/Ch-PLGA particles and the assessment of their
15 anticancer properties against lung cancer cells. The encapsulation efficiency (EE) and the
16 drug loading content (DLC) in multifunctional nanosystems for active or passive targeting are
17 important parameters that define not only the structure and the properties of the system, but
18 also its application efficiency [38, 39]. In the present study we examine the possibility of
19 using differential thermal analysis/thermogravimetric analysis (DTA/TGA) coupled with *on-*
20 *line* mass spectrometry to obtain direct information on the efficiency of the androstane
21 derivative encapsulation. The release of the drug is also assessed, knowing that the drug
22 release kinetics defines the effects that the therapy will have on both malignant and healthy
23 cells. Needless to add, anticancer drugs are toxic not only to the cancer cells, but also to the
24 healthy ones. Designing selective anticancer medicaments, having a high activity toward
25 cancer cells but a low activity toward healthy ones, has been an immense challenge in the past

1 twenty years [40, 41]. Tools that employ nanotechnologies may be successfully applied to
2 address these challenges [42, 43]. In this study we test the comparative cytotoxic response of
3 human lung carcinoma (A549) and healthy human lung fibroblasts (MRC5) to their *in vitro*
4 treatment with the A-loaded HAp/Ch-PLGA composite particles. We have also analyzed the
5 period of recovery of the two types of cells after their treatment with A-loaded HAp/Ch-
6 PLGA.

7

8 **2. Materials and methods**

9 **2.1. Synthesis of 17 β -hydroxy-17 α -picolyl-androst-5-en-3 β -yl acetate-loaded HAp/Ch- 10 PLGA**

11 **2.1.1. Synthesis of 17 β -hydroxy-17 α -picolyl-androst-5-en-3 β -yl acetate (A)**

12 The first stage in the preparation of 17 β -hydroxy-17 α -picolyl-androst-5-en-3 β -yl-acetate (A)
13 was the addition of α -picolyl lithium to the 17-oxo group of dehydroepiandrosterone, resulting
14 in 17 α -picolyl-androst-5-en-3 β ,17 β -diol. This was followed by the acetylation using acetic
15 anhydride in absolute pyridine at room temperature for 3 h [44].

16 **2.1.2. Synthesis of HAp, HAp/Ch-PLGA and A-HAp/Ch-PLGA**

17 An aqueous calcium nitrate (Ca(NO₃)₂) solution (150 ml; 26.6 wt.%) was added to the
18 solution of ammonium phosphate ((NH₄)₃PO₄) (7 ml H₃PO₄+165 ml NH₄OH+228 ml H₂O) at
19 50°C over the period of 60 minutes, while stirring at the rate of 100 rpm. The solution was
20 then subjected to a heat treatment at 100°C for 60 minutes [45]. The resulting gel was dried at
21 the room temperature in a vacuum drier for 72 h, after which the final product - HAp powder -
22 was obtained. X-ray diffraction run on the HAp powder confirmed its poorly crystalline
23 nature, whereas the PSD technique enabled us to determine the particle size of d₅₀=70 nm
24 [19].

1 Chitosan (Ch) of a low molecular weight (Aldrich, deacetylation > 75%), dissolved in
2 acetic acid (1 wt.%), was mixed with 17 β -hydroxy-17 α -picolyl-androst-5-en-3 β -yl acetate
3 powder (A) in 2:1 weight ratio while stirring with a magnetic stirrer at 400 rpm. PLGA
4 (50:50, Sigma, USA) dissolved in acetone was mixed with the A-containing Ch solution and
5 HAp gel in 2:3:5 weight ratio. A water solution of poloxamer 188
6 (polyethylenepolypropylene glycol, 0.1 vol.%) was added dropwise to the resulting mixture,
7 while stirring at 21,000 rpm. The obtained mixture of A, chitosan, PLGA and HAp was
8 slowly poured into a glutaraldehyde solution (Grade I, 25% in H₂O), while stirring at 21,000
9 rpm for 1 h. The obtained mixture was then centrifuged at 3000 rpm and 5°C for 1 h, and the
10 resulting precipitate was subjected to lyophilization at temperatures ranging from -10 to -60°C
11 and pressures ranging from 0.37 mbar to 0.1 mbar for 1 to 8h [19, 46]. The obtained powder
12 was washed with distilled water twice, centrifuged at 1000 rpm and dried again. The final
13 product was a powder composed of HAp particles coated with A-loaded chitosan-poly(D,L)-
14 lactide-co-glycolide (A-HAp/Ch-PLGA). The weight ratio of HAp:Ch:PLGA:A components
15 was 5:2:2:1.

16 **2.2. Characterization**

17 NMR spectra were recorded on a Bruker AVANCE III HD 400 spectrometer operated at 400
18 MHz (¹H) and 100 MHz (¹³C), and are reported in ppm downfield from the tetramethylsilane
19 internal standard. A thermal analysis was simultaneously performed using a Thermo-
20 Gravimetric Analysis/Differential-Thermal Analysis (TGA/DTA), SETSYS 2400 CS
21 Evolution (Setaram Instrumentation) coupled with a mass spectrometric (MS) gas analysis
22 system (Omni Star, Pfeiffer). Samples containing 10 \pm 0.5 mg were analyzed in air by heating
23 (10°C/min) from 28°C to 600°C. Differential scanning calorimetry (DSC) measurements were
24 performed on an Evo 131 (Setaram Instrumentation) differential scanning calorimeter.
25 Samples containing 10 \pm 0.5 mg were analyzed in nitrogen by heating (10°C /min) from 25°C

1 to 360°C. Infrared spectroscopy (FT-IR) was done on an Micro-FT-IR Nicolet iN10 with a
2 DTGS room T detector (Thermo Scientific Instruments), using the KBr technique in the
3 spectral range from 400 to 4000 cm^{-1} . The particle size distribution (PSD) was measured on
4 10 mg/mL of powders dispersed in water using a Mastersizer 2000 (Malvern Instruments
5 Ltd.) and a HydroS dispersion unit for liquid dispersants. Microstructural characterization was
6 performed using an atomic force microscope (AFM, Thermo Microscopes, Autoprobe CP
7 Research). Zeta potential of the particles in suspension was measured in the 1.5-13 pH range
8 on a Zetasizer Nano-ZS (Malvern) dynamic light scattering (DLS) device. Drug release
9 experiments were performed by placing the powder in 50 ml phosphate buffered saline (PBS)
10 in closed conical tubes and incubating at 37°C and 50 rpm for up to three weeks. To estimate
11 the concentration of the released drug, aliquoted solutions were analyzed in duplicates for
12 absorbance on a UV/Vis spectrophotometer (Nanodrop 2000, Thermo Scientific) at $\lambda=263$
13 nm.

14 **2.3 Cell culture**

15 Cytotoxicity assays were performed using two cell lines, human lung carcinoma (A549
16 ATCC CCL 185) and human lung fibroblasts (MRC-5 ATCC CCL 171) in Dulbecco's
17 modified Eagle's medium (DMEM, Gibco BRL, UK) with 4.5g/l glucose, 10% FBS (fetal
18 bovine serum, Sigma) and containing penicillin(100I.U./ml) and streptomycin (100 μ g/ml).
19 The cell lines were grown at 37°C and in air containing 5 % CO_2 (Heracus). The cells were
20 passaged twice a week; those used in the experiments were in the log phase of growth
21 between the third and the tenth passage. Only the viable cells were used. The number of cells
22 and their viability were determined using the dye exclusion test with 0.1% trypan blue.

23 **2.3.1. Trypan blue dye exclusion assay**

24 Cells were seeded at 2×10^5 cells/ml in 24 well plates (Center well, Falcon), on top of a film
25 made of compressed A-HAp/Ch-PLGA particles and the total concentration of the solid phase

1 equaling 17.6 ± 0.4 mg/ml, i.e., 46.3 ± 1.0 mg/cm². After the incubation period of 48 hours, the
2 cells were trypsinized, centrifuged at 200 g for 10 minutes and resuspended in DMEM. Ten μ l
3 of the cell suspension were removed and mixed with an equal amount of 0.1% trypan blue.
4 Ten μ l of the cell/trypan blue solution were pipetted onto a hemocytometer and live cells were
5 counted using an inverted light microscope (REICHERT). Cytotoxicity was expressed in
6 percentages according to the following formula: $CI = (1 - N_s/N_k) \times 100\%$, where N_k was the
7 average number of cells in the control samples, and N_s was the average number of cells in the
8 samples containing the examined substance. All the samples for the dye exclusion test were
9 analyzed in quadruplicates.

10 **2.3.2. MTT cell viability assay**

11 Both lung carcinoma cells and fibroblasts were plated at 2×10^5 cells/well in a 96-well
12 microtiter plate (Falcon) and grown on top of a film made of compressed HAp/Ch-PLGA or
13 A-HAp/Ch-PLGA particles and the total concentration of the solid phase equaling 17.6 ± 0.4
14 mg/ml, i.e., 46.3 ± 1.0 mg/cm². After 48 hours, the MTT reagent was added into all the wells
15 and incubated for 3 hours at 37°C. After 3 hours, 100 μ l of 0.04 mol/l HCl in isopropanol
16 were added to each well. Absorbance was read immediately using a microtiter plate reader
17 (Multiscan, MCC/340) at the wavelength of 540 nm and the reference wavelength of 690 nm.
18 Cytotoxicity was expressed in percentages according to the formula: $CI = (1 - A_s/A_k) \times 100\%$,
19 where A_k was the average absorbance of the control samples and A_s was the average
20 absorbance of the samples containing the examined substance. All the samples for the
21 colorimetric viability test were analyzed in quadruplicates.

22 **2.3.3. Immunofluorescent staining**

23 Primary mouse lung fibroblasts were isolated from 9-week old C57BL/6 mice. Fibroblasts
24 were grown in DMEM with 10% fetal bovine serum and 5% antibiotic
25 (penicillin/streptomycin) and antimycotic (fungazone). For the immunofluorescence analysis,

1 the cells were seeded onto glass coverslips at 1×10^5 cells per well in a standard 24-well plate
2 and 1 mg/ml of nanoparticles were added in the powder form to them and swirled to achieve
3 proper dispersion. In addition to dispersed particles, this yielded a moderate amount of
4 particle conglomerates, visually observable in interaction with the cells under a fluorescent
5 optical microscope. Cells were allowed to grow in conjunction with the particles for 4 days at
6 37°C , 5% CO_2 . On Day 4, the cells were fixed in 4% paraformaldehyde for 5 minutes, washed
7 thrice in PBS, for 5 minutes each, and stained with Alexa Fluor 568 rhodamine-phalloidin
8 (1:400) for 30 minutes. After staining, the cells were washed thrice, for 5 minutes each, in
9 PBS and incubated for 25 minutes with NucBlue Fixed Cell Ready probe reagent. Cells were
10 then rinsed thrice with PBS, for 5 minutes each, and the coverslips with cells were mounted
11 onto microscope glass slides using Prolong Diamond and cured overnight. Images were
12 acquired on a Zeiss LSM 710 confocal microscope (UIC core imaging facility).

13

14 **3. Results and discussion**

15 **3.1. ^1H NMR and ^{13}C NMR analyses of 17β -hydroxy- 17α -picolyl-androst-5-en- 3β -yl 16 acetate (A)**

17 In the ^1H NMR spectra of the androstane derivative (Fig. 1a), compound A, the signals
18 from protons from the heterocyclic ring at C-17 position were detected with a downfield shift,
19 in the 7.20-8.47 ppm range, as a result of the deshielding influence of the nitrogen atom. Two
20 protons from C-20 methylene group were registered at 2.84 and 3.11 ppm. The ^{13}C NMR
21 spectral signals (Fig. 1b) were assigned to carbons from the heterocyclic ring at 122.38,
22 124.91, 137.00, 147.78 and 160.75 ppm. As expected, the signal from 3-acetoxy group of the
23 compound A was detected in ^1H NMR spectra at 2.05 ppm. The corresponding relatively
24 intense ^{13}C NMR signals were observed at 21.46 and 170.60 ppm for methyl and carbonyl
25 atoms from the 3-acetoxy group, respectively.

1 **Fig. 1.**

2 **3.2. Differential-Thermal Analysis (DTA)**

3 The DTA thermograms of HAp, Ch, PLGA, A and A-HAp/Ch-PLGA in the
4 temperature range of 25-600°C are shown in Figure 2. In contrast to the one-step thermal
5 degradation of chitin, the thermal degradation of chitosan is characterized by a two-step
6 process entailed by chain scission and repolymerization reactions [47]. After the evaporation
7 of the surface-bound water, yielding the broad endothermic peak at 100°C (Fig. 2a.), one may
8 notice a narrow endothermic peak in the 306–320°C interval. As in agreement with previous
9 findings, this thermal event derives from the degradation and deacetylation of chitosan [48]
10 and the formation of vaporous fragments with a lower molecular weight, including char [47].
11 A sequence of endothermic peaks in the DTA thermogram of PLGA (Fig. 2a) at temperatures
12 between 328°C and 342°C, as well as two major peaks centered at 364°C and 390°C,
13 originate from the decomposition of the polymer. The decomposition of PLGA at
14 temperatures around 360°C has also been observed by other authors [49]. The suggested
15 mechanism for the thermal degradation of PLGA is based on an interaction between the end
16 hydroxyl groups of the polymeric chains and the resulting formation of cyclic oligomers,
17 lactides, acetaldehydes, methyl glycolates and carbon monoxide. At higher temperatures, the
18 release of carbon dioxide, methyl ketene or formaldehyde occurs [50, 51].

19 **Fig. 2.**

20 As seen in Fig.2b, the DTA thermogram of A-loaded HAp/Ch-PLGA is dominated by
21 peaks characteristic for the pure compound A. The two most prominent endothermic peaks of
22 the compound A, at 204 and 330°C (zone I and II in Fig.2b, respectively), are detected in A-
23 loaded HAp/Ch-PLGA too. The sequence of endothermic peaks originating from the
24 degradation PLGA is observed at the higher temperature end of the 330°C peak exhibited by
25 the compound A. The major one of these peaks exhibits a shift to lower temperatures, from

1 365 to 340°C, following the combination of PLGA with HAp in composite HAp/Ch-PLGA,
2 suggesting the surface presence of the polymeric phase in a thinner form, more exposed to the
3 atmospheric heat than the bulkier, pure PLGA particles, thus requiring lesser heat to reach
4 their interior and initiate the degradation process. The semblance of the two thermograms
5 presented in Fig.2b unequivocally indicates that the method for the encapsulation of the
6 compound A into HAp/Ch-PLGA (section 2.1.1) preserves its native structure.

7 **3.3. TG-MS Gas Analysis**

8 A comparative overview of thermogravimetric (TG) diagrams and the corresponding
9 MS spectra of the gas product analysis of the A-loaded HAp/Ch-PLGA composite and of its
10 four individual components are shown in Figure 3. The TGA curve of HAp demonstrates a
11 total weight loss of about 7% at 600°C [52], being solely due to the evaporation of physically
12 adsorbed water, which, as seen from the corresponding mass spectrogram, is completed by the
13 point the system reaches about 250°C. This observed water loss, centered at around 100°C,
14 agrees with the results of the DT analysis (Fig. 2a).

15 **Fig. 3.**

16 The moderate weight loss of chitosan (Fig. 3a) up to about 250°C is associated with
17 the loss of adsorbed water. In the subsequent 250-600°C interval, the region extending from
18 250°C to 300°C stands out with a drastic weight decrease: from 86% to 55%. It is followed by
19 the region extending from 300°C to 450°C, where a milder weight loss is observed, from 55%
20 to 30%, and then the region extending from 450 to 600°C, where the weight drops from 30%
21 down to only 0.6%. Similar TGA results for chitosan have been reported by others as well
22 [53, 54]. A dramatic chitosan weight loss in the 306–320°C temperature interval due to
23 deacetylation (Fig. 2a) is confirmed by the increasing CH₃COOH concentration in the MS gas
24 analysis. The release of H₂O, CO, CO₂ and CH₃COOH in the 280–500°C temperature interval
25 has also been confirmed by other authors by means of FT-IR analyses [53]. In the temperature

1 interval between 260 and 400°C, a sudden drop in the PLGA weight occurs (Fig. 3b) and a
2 complete degradation takes place at temperatures higher than 400°C. According to the
3 thermogravimetric analyses of PLGA reported earlier, the pyrolysis of PLGA was detected at
4 temperatures as low as 255°C, whereas its full degradation occurred at temperatures
5 extending from 400°C upwards [49, 55]. In the 260°C-400°C interval, the significant PLGA
6 weight loss is accompanied by the maximal contents of CO₂, H₂O and CH₃COOH in the MS
7 gas analysis. Physicochemical phenomena caused by heating the compound A to 600°C were
8 reflected in 98.71 % total weight loss (Fig. 3c). Detected in the 428-528°C interval, the
9 dissociation of benzene from the rest of the molecule, typical for the MS spectrum of the
10 compound A, indicates the fragmentation of its structure. The TGA curve for A-HAp/Ch-
11 PLGA shown in Figure 3d demonstrates a 56.63 % weight loss at 600°C. The remaining
12 43.37 % of weight contains only HAp and the compound A, given that the weight ratio of Ch
13 and PLGA at this final stage of the heating process is 0% due to their complete thermal
14 degradation. Every single MS spectrum of the individual constituents of the composite system
15 - HAp, Ch, PLGA and the derivative A - is present in the MS spectrum of A-HAp/Ch-PLGA,
16 which, together with the DTA results, confirms the successful encapsulation of the derivative
17 A.

18 **3.4. Fourier transform infrared spectroscopy (FT-IR) analysis of A and A-HAp/Ch-** 19 **PLGA**

20 In order to additionally confirm the qualitative content of A-HAp/Ch-PLGA, the material was
21 analyzed using FT-IR spectroscopy. The IR spectra of the compound A and of A-HAp/Ch-
22 PLGA composite particles are shown in Fig.4a. The wide IR band in the spectrum of A (17β-
23 hydroxy-17α-picolyl-androst-5-en-3β-yl acetate), with the maximum at 3303 cm⁻¹, is assigned
24 to the O-H stretch (17β-OH). Several absorption bands with maxima at 2943 and 2827 cm⁻¹

1 originate from the alkyl stretches of the C–H group ($-\text{CH}_3$ and $=\text{CH}_2$). The valence vibrations of
2 the acetate group were observed as a sharp band with the maximum at 1246 cm^{-1} .

3 The IR spectrum of A-HAp/Ch-PLGA is additionally characterized by the absorption
4 bands arising from HAp, Ch and PLGA: $\nu(\text{OH})$ $3600\text{--}3000\text{ cm}^{-1}$ (HAp, PLGA, Ch; intensive,
5 wide band), $\nu(\text{C}=\text{O})$ 1750 cm^{-1} (PLGA; strong intensity), $\nu(\text{C}=\text{O})$ 1655 cm^{-1} (amide I band,
6 Ch; strong intensity), $\delta(\text{HOH})$ approx. 1640 cm^{-1} (HAp, PLGA, Ch; weak to medium
7 intensity), $\delta_{\text{sy}}(\text{CH})$ 1380 and $\delta_{\text{as}}(\text{CH})$ 1450 cm^{-1} (HAp, PLGA, Ch; weak to medium intensity),
8 $\delta(\text{OH})$ 1420 cm^{-1} (HAp, PLGA, Ch; medium intensity), $\nu(\text{P}=\text{O})$ 1090 cm^{-1} (HAp; strong
9 intensity), $\nu(\text{P}-\text{O})$ 1025 cm^{-1} (HAp; strong intensity overlapped with a band $\nu(\text{P}=\text{O})$), $\delta(\text{CH})$
10 and $\delta(\text{OH})$ $1000\text{--}700\text{ cm}^{-1}$ (HAp, PLGA, Ch; weak intensity) [19, 45]. The existence of the
11 absorption bands originating exclusively from the compound A in the IR spectrum of A-
12 HAp/Ch-PLGA confirms that the substance was entrapped inside the composite particles.
13 Specifically, the group of absorption bands with maxima at about 2943 and 2827 cm^{-1} (C–H
14 group from $-\text{CH}_3$ and $=\text{CH}_2$), $=\text{C}=\text{O}$ with the maximum at around 1727 cm^{-1} , as well as the
15 valence vibrations of the acetate group at 1246 cm^{-1} indicate the presence of A in A-HAp/Ch-
16 PLGA.

17 The cross-linking of chitosan (Ch) by glutaraldehyde (GA) is a complex process,
18 typically leading to inhomogeneous products [56]. Mechanistically, free pendant amine
19 groups of chitosan react with the aldehydic group of GA and yield imine bonds [57],
20 distinguishable as the 1600 cm^{-1} peak in the corresponding IR spectra and originating from
21 the stretching vibration of the imine $\text{C}=\text{N}$ bond [58]. This peak was observed in Ch hydrogels
22 formed in a range of different Ch:GA weight ratios (16:1 to 1:1) [59], while the Ch:GA
23 reaction is reported to reach equilibrium at Ch:GA = 1:1 conditions [57]. In this study we
24 used GA as a cross-linker to a fivefold amount compared to the content of Ch (Ch:GA = 5:1).

1 The appearance of a new peak following the initiation of the cross-linking reaction at 1600
2 cm^{-1} (Fig.4a) indicates that the cross-linking occurred in the system.

3 **Fig. 4.**

4 **3.5. Particle size distribution and AFM analysis of A-HAp/Ch-PLGA**

5 The morphology and the size distribution of particles constituting A-HAp/Ch-PLGA powders
6 are shown in Figure 4b. The particles had a uniform distribution of sizes, with the d_{50}
7 parameter equaling 168 nm. The composite powder contained a small portion of larger and
8 smaller particles; correspondingly, d_{90} =247 nm and d_{10} =135 nm. The surface morphology of
9 A-HAp/Ch-PLGA, analyzed using AFM in a non-contact mode (Fig. 4b), was dominated by
10 oval topographic features, originating from the spherical shape of the individual particles. The
11 coating of nanoparticles of HAp (d_{50} =70 nm) with A-loaded Ch-PLGA in the centrifugal field
12 (section 2.1.2.) yielded spherical particles of a highly similar size and shape as those obtained
13 in our earlier study on the synthesis of A-free HAp/Ch-PLGA [46].

14

15 **3.6 Differential scanning calorimetry (DSC) and the drug release from A-HAp/Ch-PLGA**

17 Figure 5a shows DSC thermograms of the derivative A and of A-HAp/Ch-PLGA. The
18 endothermic phase transformation observed in the DT analysis of A at 204°C (Fig. 2b)
19 dominates the DSC thermogram with a sharp peak centered at 213.82°C (onset 211.44°C,
20 offset 415.46°C). The same phase transition is noticeable on the thermogram of A-HAp/Ch-
21 PLGA, next to (a) the broad endothermic doublet (onset 53.62°C, offset 131.52°C)
22 corresponding to PLGA and Ch transformations, and (b) the wide exothermic peak
23 corresponding to deacetylation of Ch (onset 290.86°C, offset 312.40°C). The integration of
24 the area under the endothermic peaks paralleling the phase transitions characteristic for the
25 pure and the encapsulated derivative A yielded enthalpy (ΔH) values of 69.704 and 5.207J/g,

1 respectively. Based on the comparison of ΔH for the pure and the encapsulated A, the content
2 of the encapsulated derivative A in A-HAp/Ch-PLGA could be estimated at 7.47 wt.%. Out of
3 the projected drug content of 10 wt.% (sec 2.1.1.), 7.47 wt.% was achieved. The calorimetric
4 method involving the measurement of the enthalpy of the phase transition associated with the
5 melting of the drug within the carrier has been used with success to predict the loading
6 capacity of poorly water-soluble drugs, the category to which the compound A belongs too
7 [60].

8 One of the benefits of polymeric carriers capable of entrapping the hydrophobic drug
9 internally comes from the extension of its therapeutic half-life by preventing the aggregation
10 of the free drug due to hydrophobic forces. Another advantage comes from the sustained
11 release profile, thanks to which the concentration of the drug in the blood and/or the target
12 tissue could be maintained at the therapeutically active level, avoiding the fluctuations
13 between toxic and ineffective levels that are secondary to repetitive intravenous bolus
14 administrations. One such sustained release of A from A-HAp/Ch-PLGA is seen in Fig. 5b.
15 The release follows a relatively linear pattern, with no burst release or plateauing after three
16 weeks of release.

17 **Fig. 5.**

18 **3.7. Zeta potential analysis of HAp/Ch-PLGA and A-HAp/Ch-PLGA particles**

19 At the physiological pH, the zeta potential of A-HAp/Ch-PLGA particles (-8.7 ± 0.5
20 mV) is significantly more negative than that of A-free HAp/Ch-PLGA particles (-2.8 ± 0.8
21 mV), the reason being the masking of a portion of the positively charged amine groups of
22 chitosan by the negative charged acetate and hydroxyl groups of A. In contrast, the given
23 hydroxyl and acetate groups are more resistant to protonation under acidic conditions (pHs
24 3.5 and 5.5) than the amine groups of chitosan, explaining the greater increase of zeta
25 potential in the positive range for HAp/Ch-PLGA than for A-HAp/Ch-PLGA. The isoelectric

1 point of HAp/Ch-PLGA concordantly becomes shifted to lower values, from 6.5 to 5.6,
2 following the entrapment of A.

3 **3.8. Selectivity of the *in vitro* antitumor activity of A-HAp/Ch-PLGA**

4 Derivatization of 17-picolyl androstane presents a promising starting point for the
5 development of a new group of compounds for the treatment of cancer. Modified androstane
6 derivatives exhibited an antiproliferative activity against several types of human cancer cells,
7 including breast, prostate, cervical, colon and lung adenocarcinoma [37, 61]. Here we report
8 the parallel assessment of cytotoxicity of the compound A, an androstane derivative, delivered
9 using submicron HAp/Ch-PLGA particles as a carrier, on two different cell lines: A549
10 human lung carcinoma cells and regular MRC5 human lung fibroblasts. After the incubation
11 with A-HAp/Ch-PLGA for 48h, the viability of the cells measured using the trypan blue
12 exclusion test was $83\pm 3\%$ for MRC5 fibroblasts and $65\pm 2\%$ for A549 lung carcinoma cells.
13 The viability for both cell lines was lower than for the corresponding untreated controls as
14 well as for the cells treated with A-free HAp/Ch-PLGA particles ($p < 0.05$), in which case the
15 viability equaled $89\pm 2\%$ for both cell types (data not shown). In contrast, cytotoxicity for the
16 MRC5 fibroblasts treated with A-HAp/Ch-PLGA particles was $17\pm 1\%$, whereas that for
17 A549 tumor cells was $46\pm 2\%$ (Fig. 6a). Accordingly, the cytotoxic effect of A-HAp/Ch-
18 PLGA particles is almost three times greater on the malignant A549 cell than on the healthy
19 MRC5 fibroblasts. The activity of the pure compound A was equally selective as that of A-
20 HAp/Ch-PLGA, given that the viability of A549 and MRC5 cells treated with the pure
21 compound A equaled 36 % and 83 %, respectively, at $[A] = 100 \mu\text{M}$. The corresponding IC50
22 curves are given in the Supplementary Section. When after 48 h of incubation with the tested
23 material, A549 and MRC5 cells were left to recover for another 48 h, the cytotoxicity
24 readings were $23\pm 2\%$ for A549 tumor cells and $11\pm 1\%$ for MRC-5 cells (Fig. 6b). This is to
25 say that following the treatment with A-HAp/Ch-PLGA particles, the malignant cells

1 recovered twice as fast as the healthy ones. This can be explained by the different nature of
2 these two cell lines, A549 and MRC5, with the malignant cells being more aggressive and
3 proliferating more intensely than the regular fibroblasts [62]. The opposite effect,
4 interestingly, is observed for A-free HAp/Ch-PLGA particles, in which case A549 cells did
5 not exhibit any recovery in the same period of time (Fig. 6b), as opposed to MRC5 cells,
6 which recovered almost completely, with the cytotoxicity dropping down to 2%. A similarly
7 selective behavior, targeting cancer cells more intensely than the normal cells, was previously
8 reported for drug-free HAp nanoparticles [35], the core components of composite particles
9 tested in this study.

10 The immunofluorescent staining of primary fibroblasts interacting with HAp/Ch-
11 PLGA and A-HAp/Ch-PLGA particles demonstrated no negative morphological or
12 proliferative effects on the cells (Fig. 7). No microfilament granulations were observed, and f-
13 actin fibers assume a healthy, striated form in cells in direct contact with both HAp/Ch-PLGA
14 and A-HAp/Ch-PLGA particles. No repulsion of the particles by the cells, previously
15 observed for specific Ch-HAp combinations [63], was observed either. No reduction in the
16 cell density entailed the treatment with A-free and A-loaded HAp/Ch-PLGA particles. In fact,
17 the nuclei count yielded the cell density of 35×10^3 cells/cm² for A-HAp/Ch-PLGA as opposed
18 to 24×10^3 cells/cm² for A-HAp/Ch-PLGA after 4 days of growth, suggesting that the addition
19 of A to HAp/Ch-PLGA exerted a positive proliferative effect on primary fibroblasts. The
20 main difference observed between HAp/Ch-PLGA and A-HAp/Ch-PLGA lay in the more
21 pronounced uptake of A-free HAp/Ch-PLGA. As seen in Figs. 7c and 7k, a larger number of
22 cells display the internalized particles in the population incubated with A-free HAp/Ch-
23 PLGA. The visually observed reduction in dispersability of HAp/Ch-PLGA following the
24 entrapment of A cannot stem from the modified surface charge of the particles because zeta
25 potential of A-HAp/Ch-PLGA was higher than that of HAp/Ch-PLGA at the physiological pH

1 indicating a lowered surface charge propensity for aggregation following the entrapment of
2 the drug. This effect cannot be either due to a difference in the particle size because there is
3 virtually none: d_{50} for HAp/Ch-PLGA is 168 nm and d_{50} for HAp/Ch-PLGA is 163 nm. Both
4 particles also typified by relatively narrowly dispersed spherical morphologies. An increase in
5 the hydrophobicity of the particle surface might be responsible for the larger and less
6 dispersable particle aggregates in the HAp/Ch-PLGA system containing A.

7 **Fig. 6.**

8 **Fig. 7.**

9 These data indicate the selectivity with which the androstane derivative A delivered
10 using HAp/Ch-PLGA particles imposes higher cytotoxicity on lung tumor cells than on the
11 healthy ones. The mechanism by which the given androstane derivative exerts a toxic effect
12 on the cancer cells involves the induction of the ER stress response, which results in calcium
13 release from the ER and arrest of the cell in the G1 phase cycle [37]. A carrier capable of
14 further elevating calcium levels as a result of its degradation in the lysosome, alongside
15 localizing itself in the vicinity of the ER, as HAp has been confirmed to do [35], is a logical
16 choice for the delivery vehicle for the given drug. Here we see that the therapeutic selectivity
17 of the compound A becomes preserved following its delivery by means HAp/Ch-PLGA. *In*
18 *vivo* delivery and the optimization of the drug/carrier interface will be the subjects of further
19 studies.

20

21 **4. Conclusion**

22 A novel particulate platform for targeting lung cancer and causing the death of cancer cells in
23 a selective manner was explored. Particles composed of HAp coated with the chitosan-
24 poly(D,L)-lactide-co-glycolide (Ch-PLGA) polymer blend, previously shown to localize in
25 lungs following their intravenous administration, were loaded with an androstane derivative,

1 17 β -hydroxy-17 α -picolyl-androst-5-en-3 β -yl acetate (A). The results of ¹H NMR and ¹³C
2 NMR analyses were mutually consistent, confirming the entrapment of the synthesized
3 androstane derivative inside HAp/PLGA-Ch particles in a structurally intact form. The
4 thermogravimetric and differential thermal analysis coupled with mass spectrometry was used
5 to qualitatively confirm the drug loading process. A-HAp/Ch-PLGA particles were found to
6 be spherical and had a uniform size distribution of d₅₀=168 nm. By measuring the heat flow
7 associated with phase transitions occurring during heating and calculating the corresponding
8 enthalpies, the drug content of the derivative A in A-HAp/Ch-PLGA particles was estimated
9 at 7.47 wt%.

10 The results of cytotoxicity and viability assays performed on A549 lung
11 adenocarcinoma cells and on healthy MRC5 fibroblasts demonstrated that the particles of
12 hydroxyapatite/chitosan-poly(D,L)-lactide-co-glycolide loaded with 17 β -hydroxy-17 α -
13 picolyl-androst-5-en-3 β -yl acetate exhibit almost three times higher cytotoxicity towards the
14 carcinoma cells (A549) than towards the healthy cells (MRC5). At the same time, no negative
15 effects were observed on primary lung fibroblasts, indicating the prospect with which this
16 drug delivery system could be further explored as a fine particle platform for a targeted and
17 selective cancer therapy that would impose minimal side effects on the patient.

18

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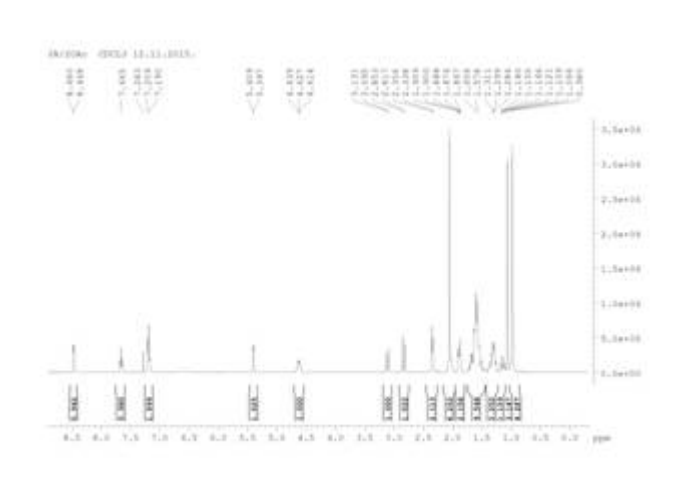
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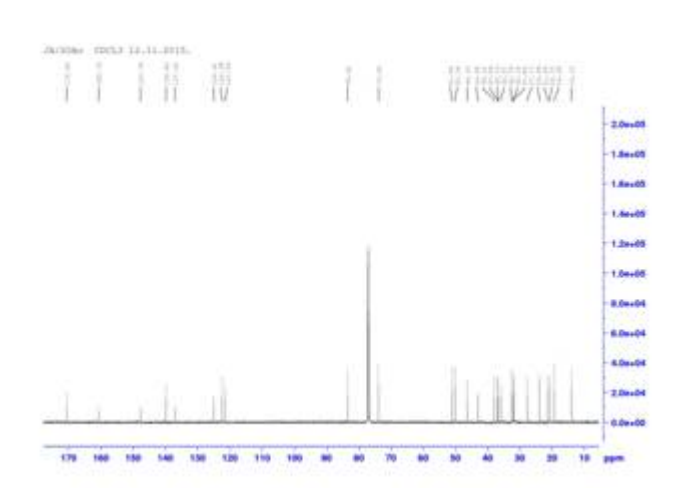
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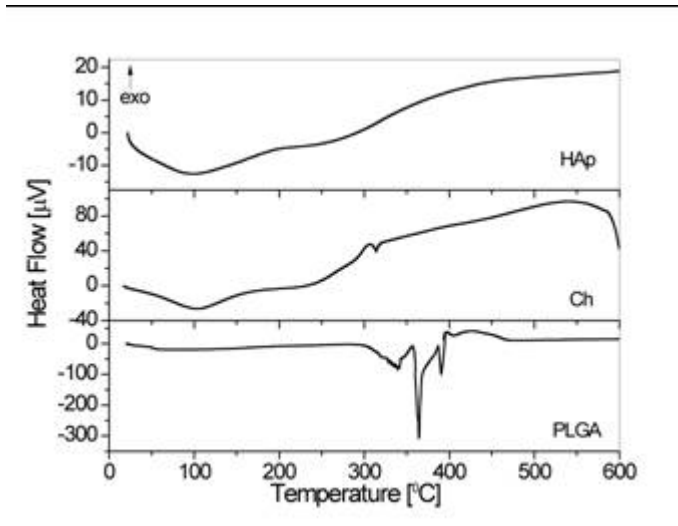


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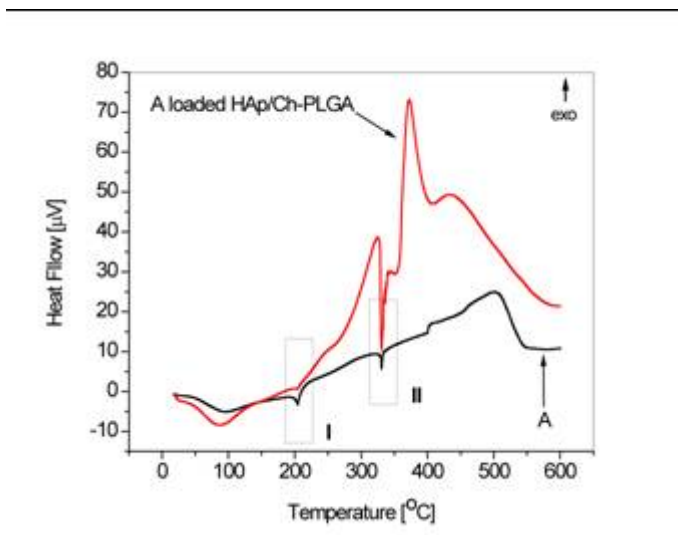
3 **Figure 1.** Nuclear magnetic resonance (NMR) spectra of 17β-hydroxy-17α-picolyl-androst-5-
 4 en-3β-yl acetate (A): a) ¹H NMR and b) ¹³C NMR. ¹H NMR (CDCl₃, δ, ppm): 0.98 (s, 3H, H-
 5 18); 1.07 (s, 3H, H-19); 2.05 (s, 3H, CH₃ from A group); 2.84 (d, 1H, J_{gem} = 14.4 Hz, CH₂Py);
 6 3.11 (d, 1H, J_{gem} = 14.4 Hz, CH₂Py); 4.63 (m, 1H, H-3); 5.40 (d, 1H, J = 4.8 Hz, H-6); 7.20
 7 (m, 2H, H-3' i H-5', Py); 7.66 (t, 1H, J_{3',4'} = J_{4',5'} = 7.8 Hz, H-4', Py); 8.47 (d, 1H, J_{6',5'} = 4.8
 8 Hz, H-6', Py). ¹³C NMR (CDCl₃, δ, ppm): 14.13 (C-18); 19.33 (C-19); 20.75; 21.46 (CH₃
 9 from A group); 23.99; 27.77; 31.84; 32.16; 32.70; 35.97; 36.73; 37.09; 38.12; 43.08; 46.37;

1 50.18; 51.06; 73.93 (C-3); 83.52 (C-17); 121.43 (C-6); 122.38 (C-5', Py); 124.91 (C-3', Py);
2 137.00 (C-4', Py); 139.80 (C-5); 147.78 (C-6', Py); 160.75 (C-2', Py); 170.60 (C=O from A
3 group).

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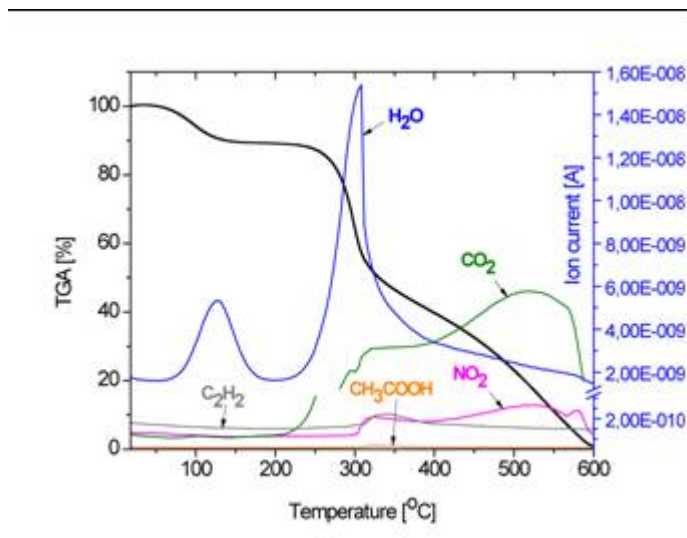


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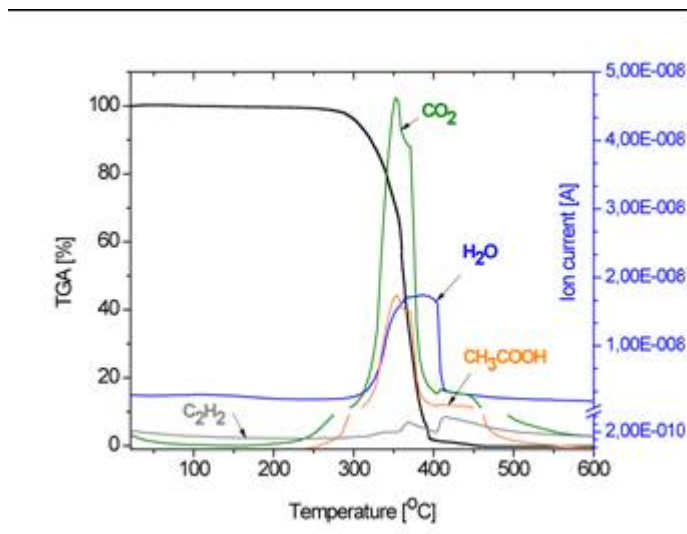


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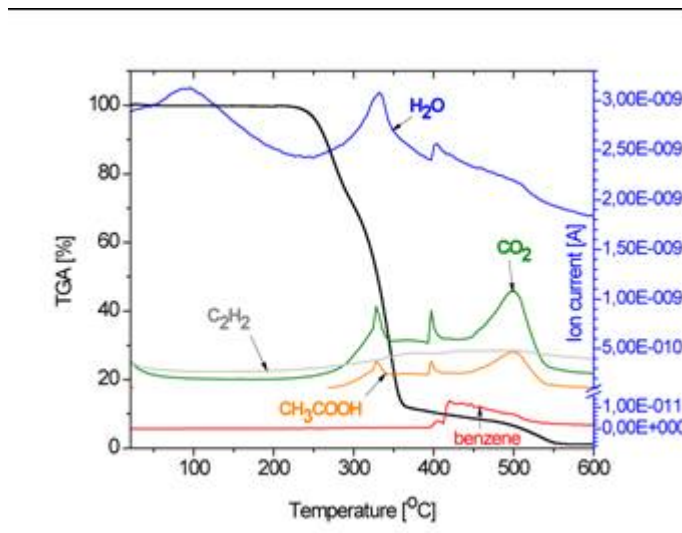
- 1 **Figure 2.** Differential thermal analysis (DTA) curves for a) HAp, Ch, PLGA and b) A and A-
- 2 HAp/Ch-PLGA



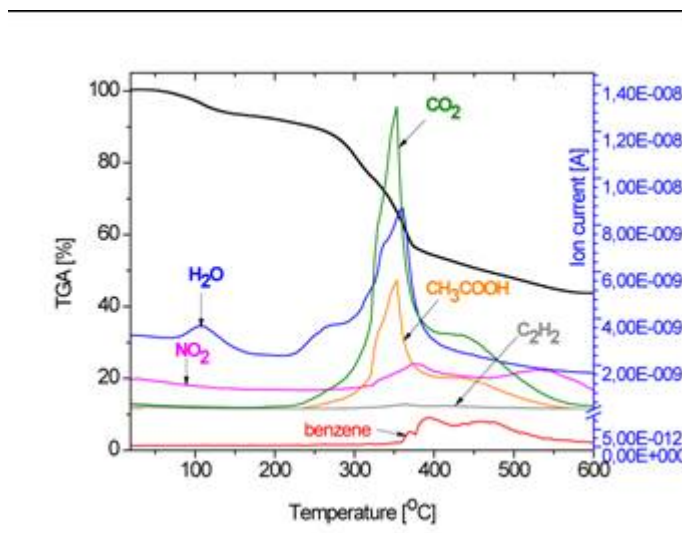
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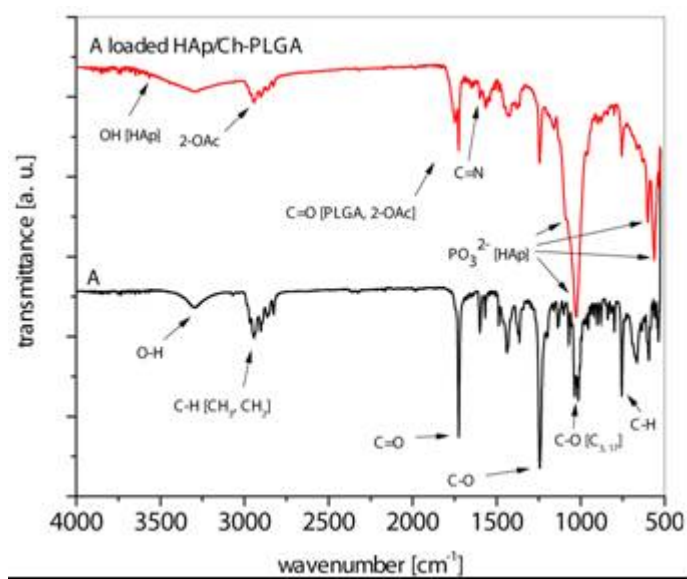


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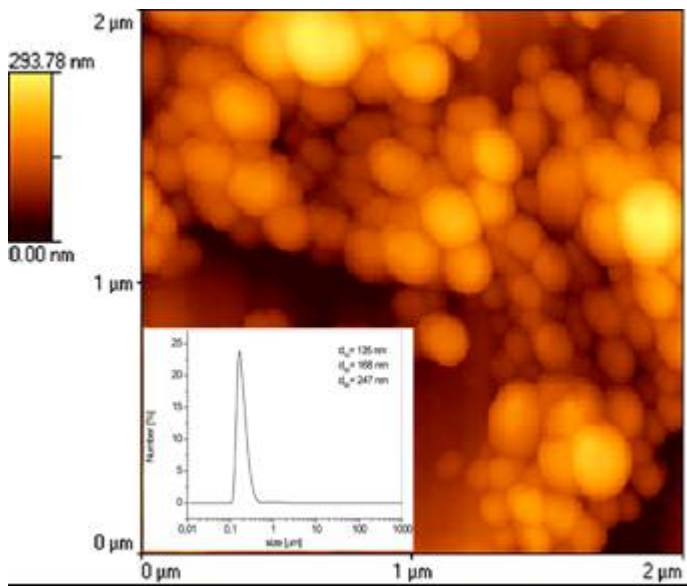


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3 **Figure 3.** Spectra obtained from the thermogravimetric (TG) and mass spectrometric (MS)
 4 gas analysis of a) Ch, b) PLGA, c) A and d) A-HAp/Ch-PLGA in the 28-600°C temperature
 5 interval and at the heating rate of 10 °C/min.

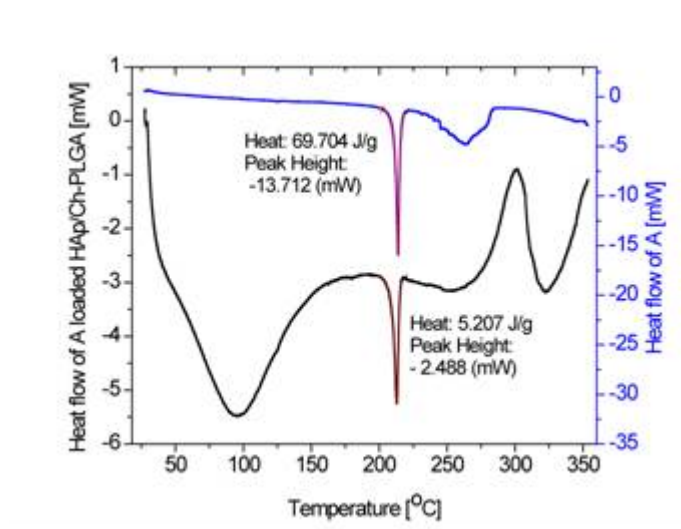


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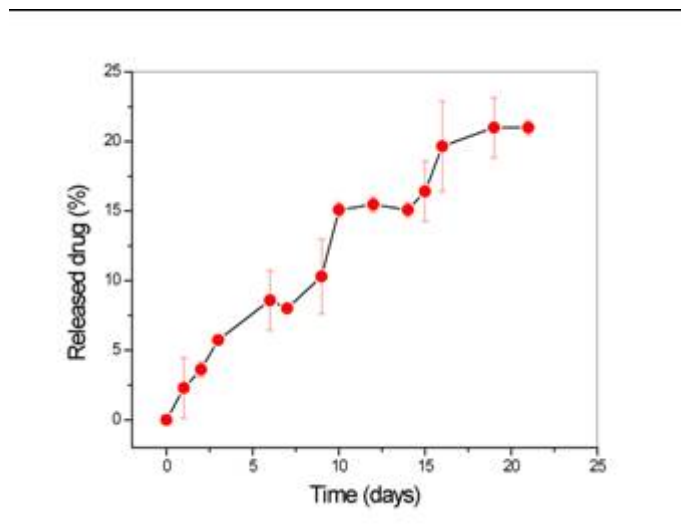


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3 **Figure 4.** IR spectroscopy and morphology of A-HAp/Ch-PLGA. a) FT-IR spectra, b) the
 4 particle size distribution and 2D AFM image

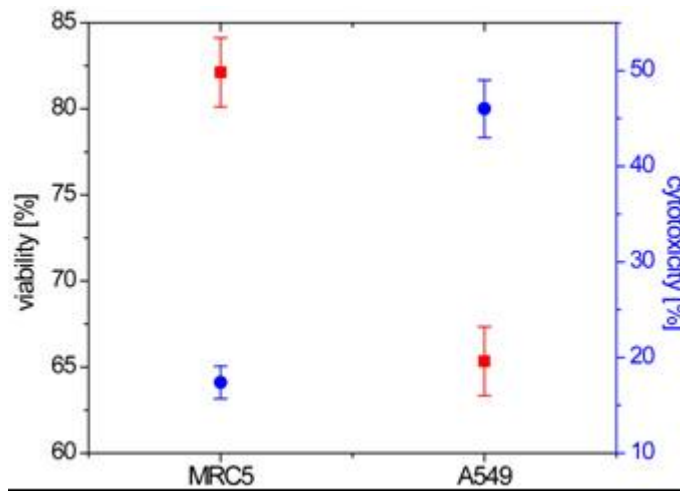


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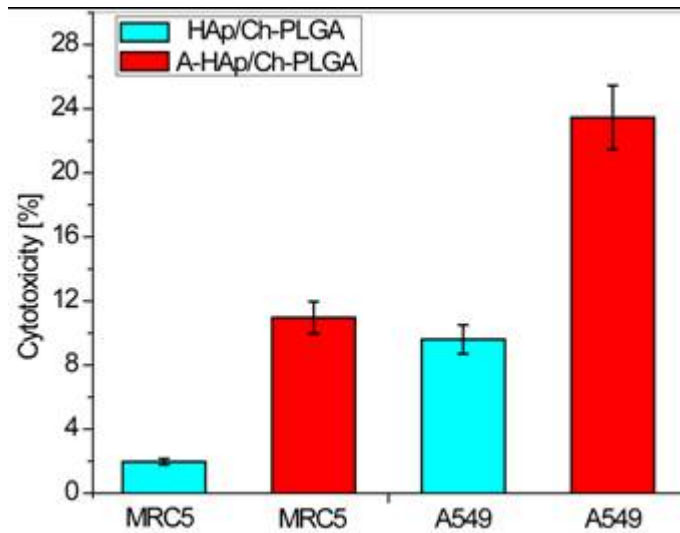


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3 **Figure 5.** a) Differential scanning calorimetry (DSC) thermograms of A and A-HAp/Ch-
 4 PLGA. b) The release of A from A-HAp/Ch-PLGA in PBS at 37 °C over the period of 21
 5 days. Data points represent averages (n=2) and the error bars represent standard deviations.

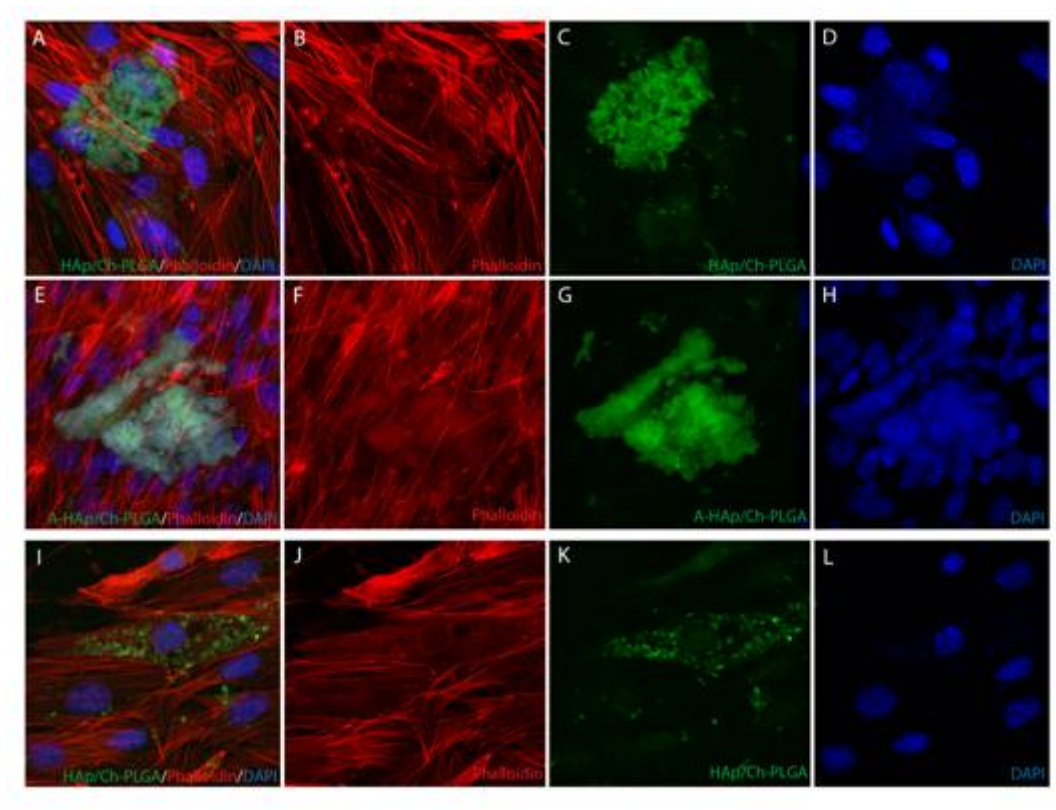


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3 **Figure 6.** Results of the *in vitro* test of A-HAp/Ch-PLGA on two different cell lines: regular
 4 MRC5 human lung fibroblasts and A549 human lung carcinoma cells: a) viability and
 5 cytotoxicity estimated using the MTT assay and the dye exclusion test, respectively; b)
 6 cytotoxicity following the 48 h recovery period. Data points represent averages (n = 4) and
 7 the error bars represent standard deviations.



1
2 **Figure 7.** Primary mouse lung fibroblasts interact with 1 mg/ml HAp/Ch-PLGA (A-D and I-
3 L) and A-HAp/Ch-PLGA particles (E-H) with no cytotoxicity or repulsion. A-D) Primary
4 mouse lung fibroblasts surround HAp/Ch-PLGA particles. E-H) Primary mouse lung
5 fibroblasts surround androstane-loaded HAp/Ch-PLGA particles. I-L) Primary mouse lung
6 fibroblasts endocytose HAp/Ch-PLGA particles. Rhodamine-phalloidin-stained f-actin (red),
7 DAPI-stained nucleus (blue), and auto-fluorescing HAp/Ch-PLGA and A-HAp/Ch-PLGA
8 particles (green).

9