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# Ligand fishing using new chitosan based functionalized Androgen Receptor magnetic particles

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ABSTRACT

Superparamagnetic nanoparticles with chemically modified chitosan has been proposed as a potential support for the immobilization of the androgen receptor (AR). The study involved comparison of different AR carriers like commercially available magnetic beads coated with silica (BcMag) and chitosan coated nanoparticles with different amount of amino groups. The immobilization was carried out through covalent immobilization of the AR through the terminal amino group or through available carboxylic acids. The initial characterization of the AR coated magnetic beads was carried out with dihydrotestosterone, a known AR ligand. Subsequently, chitosan modified nanporticles with long-distanced primary amino groups ($Fe_3O_4CS$-$(NH_2)_3$) (upto 8.34 mM/g) were used for further study to isolate known AR ligands (bicalutamide, flutamide, hydroxyflutamide and levonogestrel) from a mixture of tested compounds in ammonium acetate buffer [10 mM, pH 7.4]. The results showed that the selected nanoparticles are a promising semi-quantitative tool for the identification of high affinity compounds to AR and might be of special importance in the identification of novel agonists or antiandrogens.

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## 1. Introduction

Androgen receptor (AR) is a ligand-dependent transcription factor that controls the expression of specific genes and is a member of the nuclear receptor (NR) superfamily [1]. The mechanism by which androgens elicit their actions on different tissues has become clearer over the years. The AR protein has 3 major functional domains: a variable N-terminal domain (NTD), a highly conserved DNA-binding domain (DBD) and a conserved ligand-binding domain (LBD). Testosterone and dihydrotestosterone (DHT) are endogenous hormones which bind to the ligand-binding domain and activate AR as a transcription factor. A decrease in circulating levels of these hormones results in a decline in musculoskeletal function, increase in body fat, decrease in muscle mass and strength and recently has been identified as a risk factor for Alzheimers disease [2,3]. As a result there is a pressing need for the identification of new potential androgens. While, hormone replacement therapy is available, it is not widely used due to potential side effects. In addition to androgens, the identification of antiandrogens can be used for the treatment of prostate cancer in men and hyperandrogenism in women [4]. For this reason, the identification of antiandrogens is also of therapeutic interest. Current antiandrogens therapies are used to treat prostate cancer, include bicalutamide, flutamide and nilutamide however, these drugs have adverse effects and drug resistance [5].

Several methods have been developed for the identification of novel ligands for the AR. For example, high-throughput screening techniques, where a rapid assay is carried out to determine the biological or biochemical activity of a large number of drug-like compounds, has been used to study the interactions of endocrine disrupting chemicals with the AR [4]. As a result, HTS is useful for the discovery and identification of new selective androgen receptor modulators [6,7]. Another method developed for the identification of novel AR ligands is the AR binding-screening assays. This was also developed to characterize receptor mediated endocrine activity by measuring the inhibition of AR transcriptional activity by small molecules or measuring the blockade of ligand-binding AR [8,9]. While useful, these biochemical assays are limited by low purity and stability of functional AR protein. There are also commercially available assays, including the AR competitor assays with

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fluorescent or radiolabeled androgen ligand [10–12], however, due to the low number labeled-ligands, waste disposal and safety they have some limitation as common tools in drug discovery.

A novel technique that has been successfully used for the identification of active components from a complex mixture is ligand fishing [13–15]. In these studies, the targeted protein is immobilized onto magnetic particles surface and the resulting stationary phase is used to "fish" out potential active compounds from different mixtures [11,13,16–18]. This has been previously demonstrated with its application for the isolation of active compounds from natural products from different matrixes including plant and cell extracts [12,15,19–21].

Several types of magnetic beads have been employed for biomolecule immobilization and drug delivery [13,22]. Recently, there has been an increasing interest in magnetite (triiron tetraoxide) based micro- and nanoparticles for their potential use in different biomedical and chemical areas, because of their "flexible" surface. The chemically modified surface with inorganic or organic molecules is responsible for oxidative stabilization as well as functionalization of particles. The polycationic polymer, chitosan (CS), has recently, become an interesting material due to its non-toxicity, good mechanical properties, biocompatibility, and biodegradability [23]. As a result, CS based magnetic nanoparticles have gained increased attention as a universal carrier for drug delivery and for enzyme immobilization [24,25]. However, this polymer does not exhibit sufficient chemical stability in aqueous environment. As a result, a novel magnetite nanoparticle coated with a modified chitosan material was **s**ynthesized, specifically designed for enzyme (lipase) and protein (human serum albumin, HSA) immobilizations [26]. The resulting coated surface with chemically modified chitosan and long-distanced primary amino groups gives rise to dispersion in organic and water solvents as it is unable to form intramolecular hydrogen binding. Thus, the hydrophobic surface has significant influences on catalytic activity of the immobilized enzymes e.g lipases [27]. Hence, these nanoparticles were studied as potential magnetic supports for AR-protein immobilization.

## 2. Materials and methods

### 2.1. Materials

Androstanedione, bicalutamide, dexamethasone, dibutylphthalate, dihydrotestosterone (DHT), flutamide, hydroxyflutamide, levonorgestrel, lidocaine hydrochloride, 1-ethyl-3-(3-methylaminopropyl)carbodiimide (EDC), gluteraldehyde, hydroxylamine hydrochloride, *N*-hydroxysulfosuccinimide (Sulfo-NHS), potassium phosphate dibasic, pyridine (99.8%), sodium azide, sodium cyanoborohydride, sodium chloride, trizma base, calcium chloride, glycerol and sodium phosphate monobasic were purchased from Sigma–Aldrich (Stainhaim, Germany). HPLC grade Acetonitrile (ACN) and methanol (MeOH) were from POCh (Gliwice, Poland). Human androgen receptor (AR) full length protein was from Abcam (Cambridge, UK).

Commercially available amine terminated magnetic beads (BcMag) (50 mg/mL, 1 μm diameter) of "sophistically coated iron oxide particles to provide primary amino groups", were purchased from Bioclon Inc. (San Diego, CA, USA). A manual magnetic separator Dynal MPC-S was purchased from Invitrogen (Carlsbad, CA, USA). Solutions were prepared using purified water used in the study using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA).

### 2.2. Synthesis of chitosan magnetic nanoparticles

The 3 groups of magnetic nanoparticles were prepared based on the $Fe_3O_4$, coated with chemically modified chitosan and characterized by ATR-FTIR, 13C NMR, TGA/DTG/DSC, as previously described [21]. All of chitosan coated nanoparticles were prepared via standard co-precipitation procedure. To obtain one long amine substituent in chain, the coated chitosan was reacted with gluteraldehyde and aqueous solution of ethylenediamine. Materials containing two and three amine substituents were prepared by the reaction of chitosan with epichlorohydrin in alkali solution to form carbonyl groups which were treated with glutaraldehyde and finally with ethylenediamine [28].

The resulting magnetic materials with surface modified with long-distanced amino groups were used as a support for AR-bioligands binding. In Fig. 1 the magnetic nanoparticles ($Fe_3O_4$—CS-($NH_2$), $Fe_3O_4$—CS-$(NH_2)_2$ and $Fe_3O_4$—CS-$(NH_2)_3$) with a 1, 2 or 3 amino groups distanced from the polymer chain were presented.

### 2.3. AR immobilization onto the surface of magnetic beads

The following magnetic beads (MB) were used in AR-bioligands binding study: (a) commercially available amine-terminated magnetic beads (BcMag) with functional group density of ~250 μmol/g of MB and (b) chitosan coated MB with surface modified with long-distanced amino groups – $Fe_3O_4$—CS-($NH_2$), (c) $Fe_3O_4$—CS-$(NH_2)_2$ and d) $Fe_3O_4$—CS-$(NH_2)_3$ with the amount of free amine groups 3.15, 5.93 and 8.34 mM/g, respectively. The AR-full protein was immobilized using previously protocol with modification [16].

#### 2.3.1. Immobilization via N-terminal

The amine groups on the BcMag beads and the AR were linked by a previously described method [18], with slight modifications. Briefly, 5 mg of MB were washed with 1 mL pyridine buffer [10 mM, pH 6.0] in microcentrifuge tube. The supernatant was discarded and MB were suspended in 1 mL of 5% gluteralaldehyde and shaken for 3 h. The MB were then washed 3 times with 1 mL pyridine buffer [10 m M, pH 6.0] to remove the unreacted gluteraldehyde. 200 μL of buffer was transferred with 200 μg of full length AR protein to the MB and left under gentle rotation at 4 °C for 24 h. Next, the supernatant was discarded and 0.5 mL of glycine (1 M, pH 8) was added. The mixture was shaken for 30 min and supernatant discarded. The resulting MB was rinsed and stored in phosphate buffer [10 mM, pH 7.4] with 0.02% sodium azide. The control glycine-coated MB were made for each group of particles in the same manner but without AR protein. Finally, the 4 groups of MB were prepared by immobilization of AR protein onto the surface of MB through the amino group: (a) BcMag(NT)-AR, (b) $Fe_3O_4$—CS-($NH_2$)(NT)-AR (c) $Fe_3O_4$—CS-$(NH_2)_2$(NT)-AR and (d) $Fe_3O_4$—CS-$(NH_2)_3$(NT)-AR.

#### 2.3.2. Immobilization via COOH— group

For the immobilization of the —COOH group, we followed a previously published method with slight modifications [16]. Briefly, 5 mg of MB after the rinsing with 1 mL of MES [100 mM, pH 5.5] in a microcentrifuge tube were suspended in 300 μL of rinsing buffer, 200 μg of full length AR protein and 50 μL of a mixture of 10 mg of EDC and 15 mg of sulfo-NHS in 1 mL of water. The mixture was vortex-mixed and left for 3 h at 4 °C with gentle rotation. Next, the 20 μL of 1 M hydroxylamine was added to the final reaction and left for 3 h at 4 °C with gentle rotation. The supernatant was discarded and the MB with immobilized AR protein were rinsed 3 times with 1 mL of phosphate buffer [10 mM, pH 7.4] containing 0.02% sodium azide. The control hydroxylamine-coated MB were made for each group of particles in the same manner but without AR protein. Finally, the 4 groups of MB were prepared by immobilization of AR protein onto the surface of MB through the carboxy group: BcMag(C)-AR, $Fe_3O_4$—CS-($NH_2$)(C)-AR (c) $Fe_3O_4$—CS-$(NH_2)_2$(C)-AR and (d) $Fe_3O_4$—CS-$(NH_2)_3$(C)-AR.

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