



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 nanoparticles with long-distanced primary amino groups ($\text{Fe}_3\text{O}_4\text{CS}-(\text{NH}_2)_3$) (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 synthesized, 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

Androstenedione, bicalutamide, dexamethasone, dibutylphthalate, dihydrotestosterone (DHT), flutamide, hydroxyflutamide, levonorgestrel, lidocaine hydrochloride, 1-ethyl-3-(3-methylaminopropyl)carbodiimide (EDC), glutaraldehyde, 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 (Stainheim, 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 μ m diameter) of “sophistically coated iron oxide particles to provide primary amino groups”, were purchased from Biocon 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 Fe₃O₄, coated with chemically modified chitosan and char-

acterized 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 glutaraldehyde 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 (Fe₃O₄–CS–(NH₂), Fe₃O₄–CS–(NH₂)₂ and Fe₃O₄–CS–(NH₂)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 μ mol/g of MB and (b) chitosan coated MB with surface modified with long-distanced amino groups – Fe₃O₄–CS–(NH₂), (c) Fe₃O₄–CS–(NH₂)₂ and d) Fe₃O₄–CS–(NH₂)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% glutaraldehyde and shaken for 3 h. The MB were then washed 3 times with 1 mL pyridine buffer [10 mM, pH 6.0] to remove the unreacted glutaraldehyde. 200 μ L of buffer was transferred with 200 μ g 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(NH₂)-AR, (b) Fe₃O₄–CS–(NH₂)(NH₂)-AR (c) Fe₃O₄–CS–(NH₂)₂(NH₂)-AR and (d) Fe₃O₄–CS–(NH₂)₃(NH₂)-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 μ L of rinsing buffer, 200 μ g of full length AR protein and 50 μ L 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 μ L 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(CO₂H)-AR, Fe₃O₄–CS–(NH₂)(CO₂H)-AR (c) Fe₃O₄–CS–(NH₂)₂(CO₂H)-AR and (d) Fe₃O₄–CS–(NH₂)₃(CO₂H)-AR.

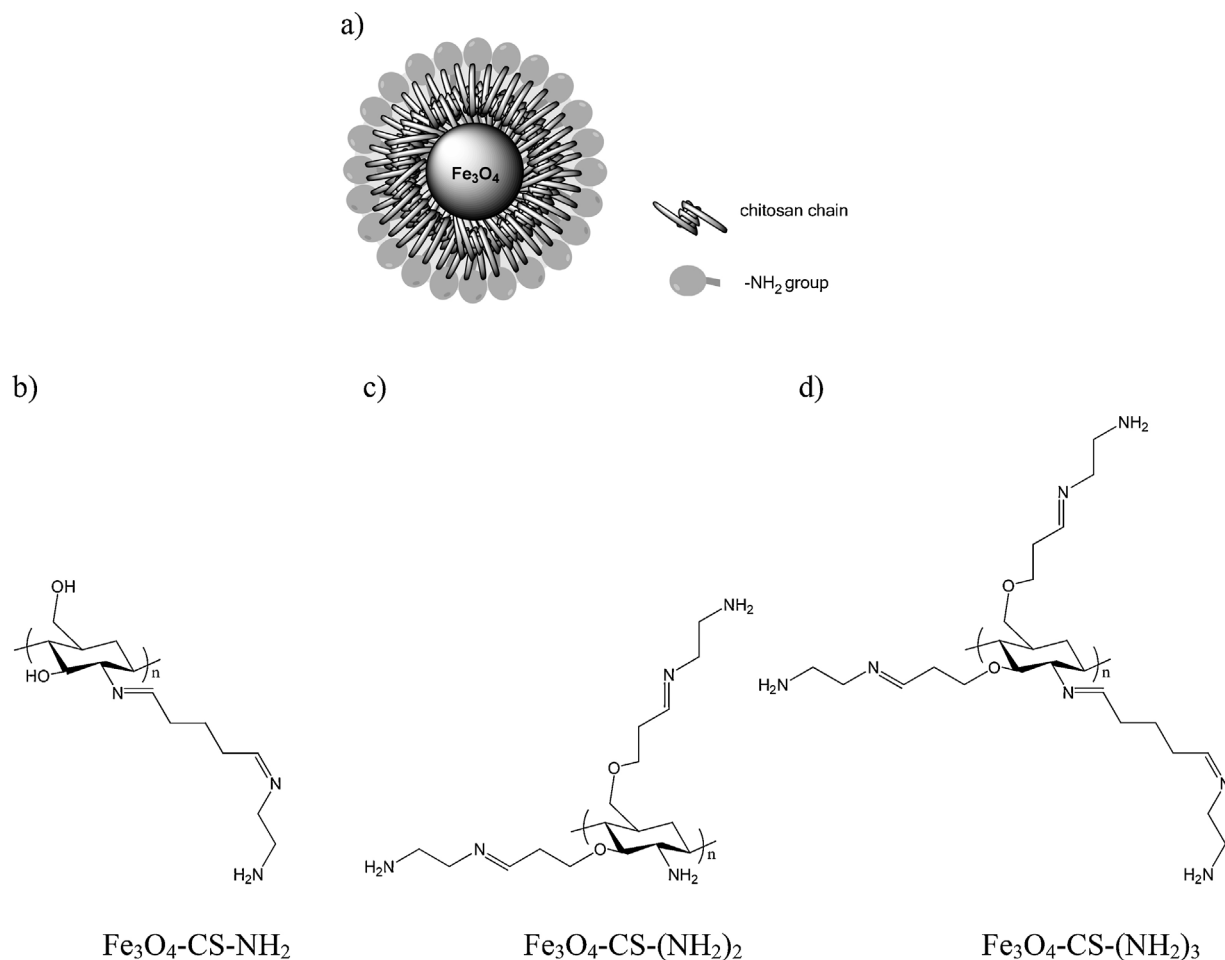


Fig. 1. Structure of prepared $\text{Fe}_3\text{O}_4\text{CS}$ nanoparticles (a) and different modifications of chitosan: with a 1 (b), 2 (c) or 3 (d) amino groups distanced from the polymer chain.

2.4. Ligand fishing

5 mg of the AR coated beads or control was suspended in a microcentrifuge tube with 10 nM of the selected drugs (Table 1) and incubated in 500 μL of ammonium acetate buffer [10 mM, pH 7.4] individually. The tube was mixed for 2 min and then placed for magnetic separator for 30 s. The first supernatant (S1) including non-binding compounds was collected. The beads were then washed with 500 μL of ammonium acetate buffer [10 mM, pH 7.4] for 2 min and the supernatant (S2) was separated and collected. The bound ligands were then eluted with 500 μL of elution buffer (ammonium acetate buffer [10 mM, pH 7.4] containing 20% methanol (v/v) twice for 2 min (S3 and S4). The magnetic beads washing procedure between extractions consisted of a double wash with 500 μL of ammonium acetate buffer [10 mM, pH 7.4] for 2 min. The binding tests were performed individually for each compound and to an equal mixture of DHT, bicalutamide, dexamethasone and lidocaine.

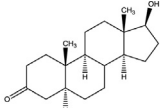
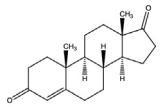
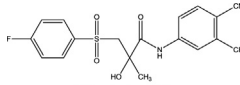
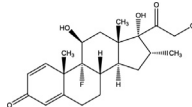
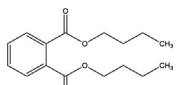
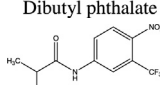
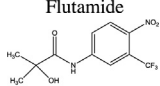
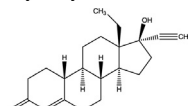
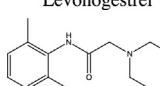
The collected supernatants were analyzed by Shimadzu UPLC Nexera X2 system consisting of: LC-30AD pump with Kinetex C18, 150 \times 4.6 mm, 2.6 μ , 100A column, SIL-30AC autosampler, CTO-20AC column oven, FCV-20-AH2 valve unit and DGU-20A5R degasser coupled with Shimadzu 8030 ESI-MS/MS triple quadrupole mass spectrometer. Mobile phase consisted of (A) 0.1% formic acid in methanol (v/v) and (B) 0.1% formic acid in water (v/v). Proportions between solvent A and solvent B were 60–40. Flow rate was set to 0.8 $\text{mL}\cdot\text{min}^{-1}$, chromatographic temperature at 35 $^\circ\text{C}$ with monitored pressure below 500 bar. Source and collision

energies as well as fragmentor voltages were optimized for each analyte by infusing a pure standard of each compound dissolved in 50% methanol. Source conditions were set to: nebulizing gas flow 1.5 L/min, drying gas flow 15.0 L/min, DL temperature 250 $^\circ\text{C}$, heat block temperature 400 $^\circ\text{C}$. Injection volume, precursor and products ions, ionization mode of each compound are presented in Table 1. All analyses of supernatant were done after incubation process in triplicate. The semi-quantification was carried out by means of signal ratio of incubated ligands in supernatant to signal of pure standard. The calculated standard curves for all tested compounds were linear with correlation coefficients greater than 0.9932 with a linear range from 0.1 nM to 10 nM.

3. Results and discussion

Covalent immobilization of the AR-protein was performed on the surface of amine terminated magnetic beads by the formation of the amide bond ((C)-AR) or reductive amination ((NT)-AR) of a carboxyl group or the amino terminal, respectively. The AR-protein was immobilized onto 4 different types of iron-oxide multifunctional magnetic nanomaterial by 2 linkers, resulting in 8 types of AR-protein magnetic beads (4 (NT)-AR and 4 (C)-AR) (section 2.3). In order to determine which AR coated magnetic particle resulted in more efficient immobilization, 10 nM of dihydrotestosterone (DHT), a known AR ligand, was incubated with each of the 8 magnetic particles (4C and 4 NT) versus control for 2 min in buffer accordance with protocol of ligand fishing (section 2.4) and analyzed by ESI-MS/MS mass spectrometer. For the control beads, the non-specific bind-

Table 1
Structural formulas, activity and analytical parameters of studied compounds.

Compound	Activity/function	Injection volume (μL)	Ionization mode	Precursor [m/z]	Products [m/z]
 Dihydrotestosterone (DHT)	androgen	10	(+)	291.3	255.3 273.3
 Androstenedione	androgen	1	(+)	287.2	109.1123.0263.3
 Bicalutamide	anti-androgen	1	(-)	429.0	255.0185.0184.0
 Dexamethasone	anti-androgen	3	(+)	393.2	373.3147.0237.1
 Dibutyl phthalate	anti-androgen	10	(+)	279.2	149.0205.1121.0
 Flutamide	anti-androgen	5	(-)	275.1	201.9205.0186.0
 Hydroxyflutamide	anti-androgen	1	(-)	291.0	205.0175.0155.0
 Levonogestrel	androgen	10	(+)	313.3	109.1245.3135.0
 Lidocaine	non-binder	5	(+)	234.6	58.1 86.1

ing for a 2 min incubation with DHT resulted in a similar range of unbound DHT in the supernatant (85.9–90.2%) and bound DHT (S3 + S4) <3% for all types of studied magnetic beads. For the (C)-AR magnetic particles, no difference was observed between the (C)-AR and control magnetic beads (data not shown), with the levels of unbound ligand similar for all the (C)-AR and control particles studied. Similarly for 3 of the 4 NT-AR (Fig. 2a) and for all 4 controls (Fig. 2b) the levels of unbound ligand (DHT) in S1 ranged from 80 to 90%, with the exception of the $\text{Fe}_3\text{O}_4\text{-CS-(NH}_2)_3\text{(NT)-AR}$ magnetic particles, where the levels of supernatants (unbound ligand) decreased to 62.3% (Fig. 2a). Of interest, when comparing the chitosan magnetic particles for the 4-NT-ARs, was that with increasing concentrations of free amino group, an increase in retained DHT was observed. For example, increasing the amount of free amino groups from 3.15 mM/g ($\text{Fe}_3\text{O}_4\text{-CS-(NH}_2)_1$) to 5.93 mM/g ($\text{Fe}_3\text{O}_4\text{-CS-(NH}_2)_2$), resulted in a significant increase of retained DHT from 5% to 10% (S3 + S4) ($p < 0.05$) and a decrease in unbound ligand (S1) from ~90% to 80%, respectively. Similarly, increasing the free amino groups to 8.34 mM/g ($\text{Fe}_3\text{O}_4\text{-CS-(NH}_2)_3$), resulted in a significant drop in unbound ligand (S1) to ~60% and a corresponding increase in retained DHT to ~20% ($p < 0.0001$). These results lead to the assumption that the higher amount of free amino

groups results in a higher yield of AR immobilization via the N-terminus. Similar observations were described previously with the use of nanoparticles as a support for the covalent immobilization of lipase and human serum albumin [21]. The results indicate that magnetic nanoparticles coated with chemically modified chitosan with an increase in free long-distanced amino groups can improve the immobilization process of biomolecules. The result of inactivity of the (C)-AR is not surprising, as the AR contains multiple surface carboxylic acids that are solvent accessible for EDC-mediated crosslinking, while the (NT)-AR is specific to the N-terminal of the protein. This non-specificity in the (C)-AR, may result in the immobilization of the protein that may block access to the ligand binding site, or cause steric effects that may prevent the binding of known ligands. The determination of the optimal crosslinker for new proteins and its molar ratios for reactions is often empirically determined.

As a result, the $\text{Fe}_3\text{O}_4\text{CS-(NH}_2)_3\text{(NT)-AR}$ nanoparticles were used for all the ligand fishing studies. The selected magnetic beads were incubated with a series of known compounds with different affinities to AR (Fig. 3). The results demonstrate that the high affinity binders, bicalutamide, flutamide, hydroxyflutamide and levonogestrel, were selectively retained on the $\text{Fe}_3\text{O}_4\text{CS-}$

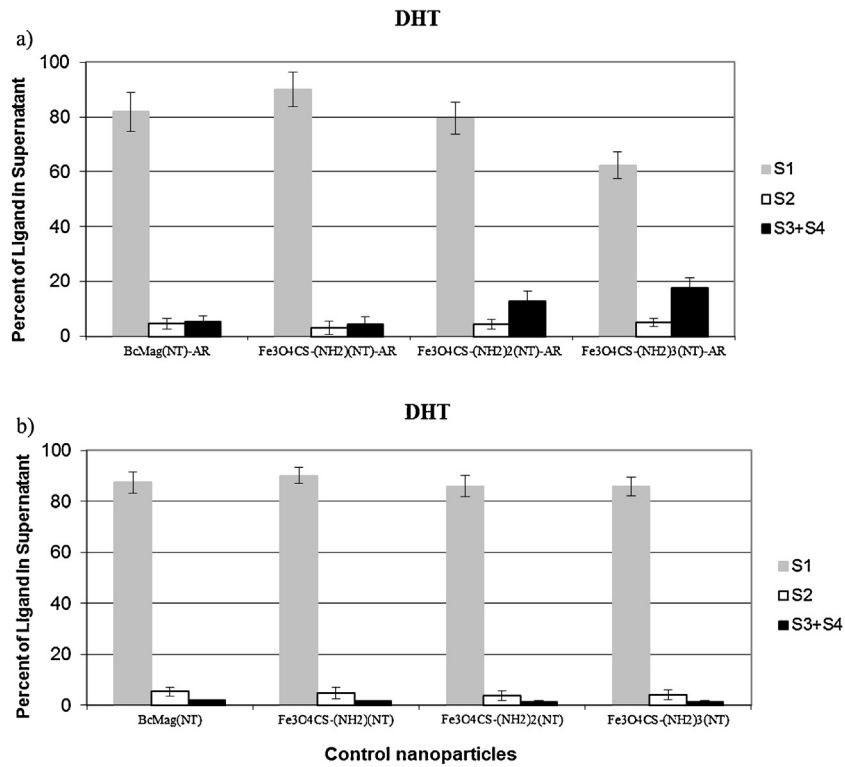


Fig. 2. Comparison of dihydrotestosterone (DHT) binding to different magnetic nanoparticles with a) immobilized AR-protein via amine group and b) control nanoparticles; S1-S4—supernatants described in ligand fishing procedure.

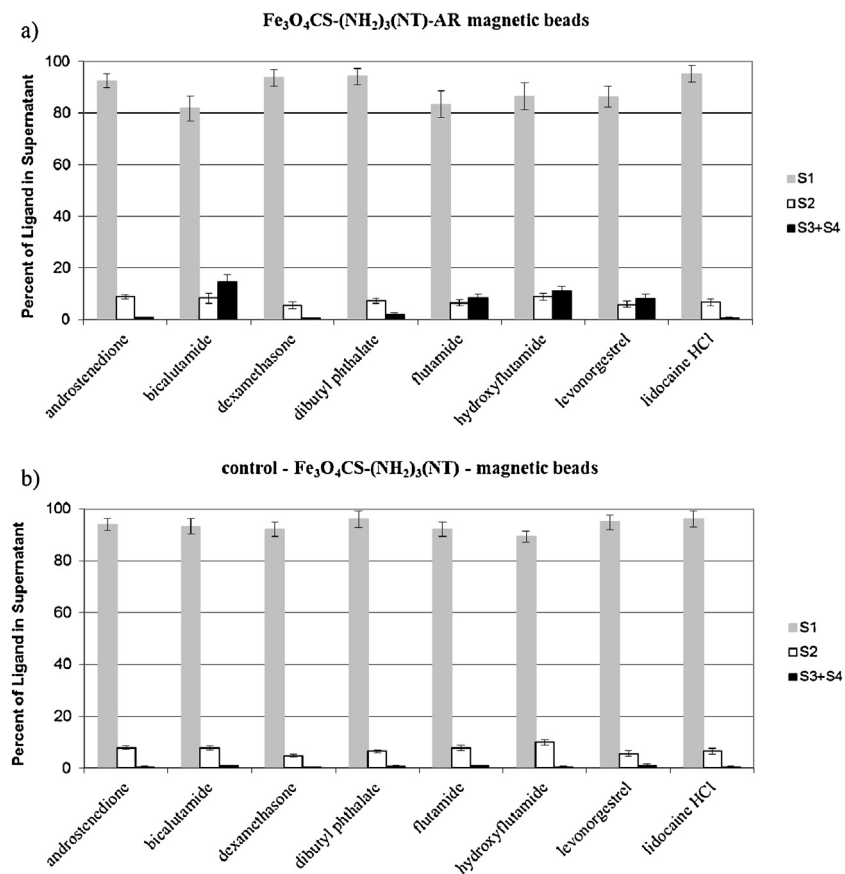


Fig. 3. Comparison of binding of androstenedione, bicalutamide, dexamethasone, dibutyl phthalate, flutamide, hydroxyflutamide, levonorgestrel and lidocaine HCl to a) Fe₃O₄CS-(NH₂)₃(NT)-AR magnetic beads and b) control nanoparticles; S1-S4—supernatants described in ligand fishing procedure.

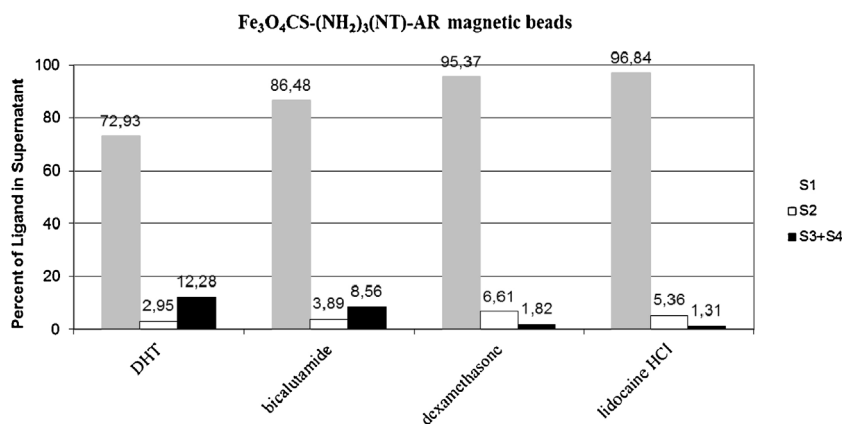


Fig. 4. The mixture ligand fishing of DHT, bicalutamide, dexamethasone and lidocaine HCl with the use of Fe₃O₄CS-(NH₂)₃(NT)-AR magnetic beads; S1-S4—supernatants described in ligand fishing procedure.

Table 2

The comparison values of binding parameters reported in literature and measured in presented study.

Compound	Relative binding affinity ^a (nM)	IC ₅₀ ^b (uM)	% of ligand binding ^c
Androstenedione	640	–	0.93
Bicalutamide	–	12.0	14.76
Dexamethasone	–	188.5	0.53
DHT	10	0.057	17.60
Dibutylphthalate	0.0017	–	1.75
Flutamide	0.0082	73.4	8.37
Hydroxyflutamide	–	33.0	11.02
Levonogestrel	11.87	–	8.05
Lidocaine	–	–	0.65

^a Relative binding affinities (recombinant rat LBD) [7].

^b IC₅₀ measured with Scintillation Proximity Assay (recombinant rat LBD) [8].

^c Percent of ligand binding–signal ratio of incubated ligands in supernatant to signal of pure standard.

(NH₂)₃(NT)-AR magnetic beads versus control (Fig. 3). Further, incubation of Fe₃O₄CS-(NH₂)₃(NT)-AR magnetic beads with other compounds (androstenedione, dexamethasone, dibutylphthalate, lidocaine), resulted in no retention in S3 and S4, indicating that these compounds were either weak binders or non-binders. In Table 2, a clear difference is seen by the % of ligand retained (S3+S4) between weak binders/non-binders and strong binders (0.96 ± 0.54)% vs (11.97 ± 4.14)%, respectively (p = 0.0012). Further, a linear trend was found between reported IC₅₀ values and the amount of retained ligand (y = 0.838 + 15.601x, r² = 0.9477), indicating that Fe₃O₄CS-(NH₂)₃(NT)-AR magnetic beads are a viable option for screening of complex mixtures to isolate and identify novel androgens or anti-androgens. In order to determine whether the beads were able to sort binders from weak/non-binders simultaneously, a mixture of DHT, bicalutamide, dexamethasone and lidocaine was incubated with the Fe₃O₄CS-(NH₂)₃(NT)-AR nanoparticles (Fig. 4). As was clearly demonstrated, the Fe₃O₄CS-(NH₂)₃(NT)-AR nanoparticles had correctly retained DHT and bicalutamide with 12% and 9% being bound (S3+S4), respectively, and with less than 2% of dexamethasone and lidocaine, weak binders, being retained, similar to control. While, some of the compounds that were not retained by the AR-coated magnetic beads, were not reported to be non-binders, the literature data on whether a compound is a weak AR-binder or non-binder is conflicting. Currently, the recombinant AR binding assay is the most commonly used method for determining the activity of endocrine active compounds, in this method the data reported is most often relative to the applied conditions and as a result is difficult to compare to classic binding assay. As a result, based on the reported AR binding

properties of the tested compounds, in terms of relative ranking of binding affinities of strong, weak and no-marginal affinity [8], our method is only capable of distinguishing high affinity binders from weak binders or non-binders (bound less than 2%).

The reported results confirm that magnetic nanoparticles might be of special importance in screening for new potential ligands. The type of coating of Fe₃O₄ core has the significant impact on the effective immobilization of biomolecules. For AR-protein the new multifunctional magnetic nanomaterial with chemically modified chitosan (Fe₃O₄–CS-(NH₂)₃) was demonstrated to be effective in fishing out ligands with high affinity for the AR from a mixture of high affinity and weak/non-binders. The subsequent development of ligand fishing technique based on the magnetic beads should be focused on the chemical and physical modification of surface of nanoparticles. This can lead to more productive and effective tool in new potential drug screening, even with weak binding to targeted protein.

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References

- [1] M.H. Eileen Tan, J. Li, H.E. Xu, K. Melcher, E. Yong, Androgen receptor: structure, role in prostate cancer and drug discovery, *Acta Pharmacol. Sin.* 36 (2015) 3–23.
- [2] J.M. Kaufman, A. Vermeulen, The decline in androgen levels in elderly men and its clinical and therapeutic implication, *Endocr. Rev.* 26 (2005) 833–876.
- [3] E.R. Rosario, C.J. Pike, Androgen regulation of beta-amyloid protein and the risk of Alzheimer's disease, *Brain Res.* 57 (2008) 444–453.
- [4] Y. Chena, N.J. Clegga, H.I. Scherb, Antiandrogens and androgen depleting therapies in prostate cancer: novel agents for an established target, *Lancet Oncol.* 10 (2009) 981–991.
- [5] S. Kojima, H. Suzuki, K. Akakura, M. Shimbo, T. Ichikawa, H. Ito, Alternative antiandrogens to treat prostate cancer relapse after initial hormone therapy, *J. Urol.* 171 (2004) 679–683.
- [6] F. Nique, S. Hebbe, C. Peixoto, D. Annot, J.M. Lefrançois, E. Duval, L.L.N. Michoux Triballeau, J.M. Lemoullac, P. Mollat, M. Thauvin, T. Prangé, D. Minet, P. Clément-Lacroix, C. Robin-Jagerschmidt, D. Fleury, D. Guédin, P. Deprez, Discovery of diarylhydantoin as new selective androgen receptor modulators, *J. Med. Chem.* 55 (2012) 8225–8235.
- [7] F. Nique, S. Hebbe, N. Triballeau, C. Peixoto, J.M. Lefrançois, H. Jary, L. Alvey, M. Manioc, C. Housseman, H. Klaassen, K. Van Beeck, D. Guédin, F. Namour, D. Minet, E. Van der Aar, J. Feyen, S. Fletcher, R. Blanqué, C. Robin-Jagerschmidt, P. Deprez, Identification of a 4-(hydroxymethyl)diarylhydantoin as a selective androgen receptor modulator, *J. Med. Chem.* 55 (2012) 8236–8247.
- [8] A. Freyberger, H.J. Ahr, Development and standardization of a simple binding assay for the detection of compounds with affinity for the androgen receptor, *Toxicology* 195 (2004) 113–126.

- [9] A. Freyberger, M. Weimer, H.S. Tran, H.J. Ahr, Assessment of a recombinant androgen receptor binding assay: initial steps towards validation, *Reprod. Toxicol.* 30 (2010) 2–8.
- [10] C. Féau, L.A. Arnold, A. Kosinski, R.K. Guy, A high-throughput ligand competition binding assay for the androgen receptor and other nuclear receptors, *J. Biomol. Screen.* 1 (2009) 43–48.
- [11] T.C. Turek-Etienne, E.C. Small, S.C. Soh, T.A. Xin, P.V. Gaitonde, E.B. Barrabee, R.F. Hart, R.W. Bryant, Evaluation of fluorescent compound interference in 4 fluorescence polarization assays, *Biomol. Screen.* 2 (2003) 176–184.
- [12] J.S. Nichols, D.J. Parks, T.G. Conser, S.G. Blanchard, Development of a scintillation proximity assay for peroxisome proliferator-activated receptor gamma ligand binding domain, *Anal. Biochem.* 257 (1998) 112–119.
- [13] K.L. Vanzolini, Z. Jiang, X. Zhang, L.C.C. Vieira, A.G. Corrêa, C.L. Cardoso, Q.B. Cass, R. Moaddel, Acetylcholinesterase immobilized capillary reactors coupled to protein coated magnetic beads: a new tool for plant extract ligand screening, *Talanta* 116 (2013) 647–652.
- [14] F. Wiekhorst, U. Steinhoff, D. Eberbeck, L. Trahms, Magnetorelaxometry assisting biomedical applications of magnetic nanoparticles, *Pharm. Res.* 29 (May (5)) (2012) 1189–1202.
- [15] S.G. Wubshet, I.M. Brighente, R. Moaddel, D. Staerk, Magnetic ligand fishing as a targeting tool for HPLC-HRMS-SPE-NMR: α -glucosidase inhibitory ligands and alkylresorcinol glycosides from *Eugenia catharinae*, *J. Nat. Prod.* 78 (11) (2015) 2657–2665.
- [16] N. Singh, S. Ravichandran, K. Spelman, S.D. Fugmann, R. Moaddel, The identification of a novel SIRT6 modulator from *Trigonella foenum-graecum* using ligand fishing with protein coated magnetic beads, *J. Chromatogr. B* 968 (2014) 105–111.
- [17] R. Moaddel, M.P. Marszał, F. Bigli, Q. Yang, X. Duan, I.W. Wainer, Automated ligand fishing using human serum albumin-coated magnetic beads, *Anal. Chem.* 79 (2007) 5414–5417.
- [18] M.C. de Moraes, K.L. Vanzolini, C.L. Cardoso, Q.B. Cass, New trends in LC protein ligand screening, *J. Pharm. Biomed. Anal.* 87 (2014) 155–166.
- [19] M.P. Marszał, A. Buciński, S. Kruszewski, B. Ziolkowska, A new approach to determine camptothecin and its analogues affinity to human serum albumin, *J. Pharm. Sci.* 100 (2011) 1142–1146.
- [20] Q. Liu, S. Shi, L. Liu, H. Yang, W. Su, X. Chen, Separation and purification of bovine serum albumin binders from *Fructus polygoni orientalis* using off-line two-dimensional complexation high-speed counter-current chromatography target-guided by ligand fishing, *J. Chromatogr. A* 1304 (2013) 183–193.
- [21] M.P. Marszał, R. Moaddel, S. Kole, M. Gandhari, M. Bernier, I.W. Wainer, Ligand and protein fishing with heat shock protein 90 coated magnetic beads, *Anal. Chem.* 80 (2008) 7571–7575.
- [22] J.L. Corchero, A. Villaverde, Biomedical applications of distally controlled magnetic nanoparticles, *Trends Biotechnol.* 27 (2009) 468–476.
- [23] N. Mati-Baouche, P.H. Elchinger, H. de Baynast, G. Pierre, C. Delattre, P. Michaud, Chitosan as an adhesive, *Eur. Polym. J.* 60 (2014) 198–212.
- [24] S. Kumar, A.K. Jana, I. Dhamija, M. Maiti, Chitosan-assisted immobilization of serratiopeptidase on magnetic nanoparticles: characterization and its target delivery, *J. Drug Target.* 22 (2014) 123–137.
- [25] S.A. Alvarez, A.I. Iyina, K.M. Jauregui, J.L. Hernandez, E.P. Ceniceros, A.Z. Cruz, H.S. Caballero, R.G. Campos, Isolation and immobilization of influenza virus-specific N-SA- α -2,3-Gal receptors using magnetic nanoparticles coated with chitosan and Maackia amurensis lectin, *Appl. Biochem. Biotechnol.* 174 (2014) 1945–1958.
- [26] M. Ziegler-Borowska, D. Chełminiak, T. Siódmiak, A. Sikora, M.P. Marszał, H. Kaczmarek, Synthesis of new chitosan coated magnetic nanoparticles with surface modified with long-distanced amino groups as a support for bioligands binding, *Mater. Lett.* 132 (2014) 63–65.
- [27] M. Ziegler-Borowska, T. Siódmiak, D. Chełminiak, A. Cyganiuk, M.P. Marszał, Magnetic nanoparticles with surfaces modified with chitosan-poly[N-benzyl-2-(methacryloxy)-N,N-dimethylethanaminium bromide] for lipase immobilization, *Appl. Surf. Sci.* 288 (2014) 641–648.
- [28] M. Ziegler-Borowska, D. Chełminiak, H. Kaczmarek, A. Kaczmarek-Kędziera, Effect of side substituents on thermal stability of the modified chitosan and its nanocomposites with magnetite, *J. Therm. Anal. Calorim.* (2016) 1–14.