IJL, Issue 2, Vol. 23, 2024
https://doi.org/10.31900/ijl.v23i2.449

Research Article

Diagnosis of Polycystic Ovary Syndrome using Free Testosterone levels via Surface Enhanced Raman Spectroscopy Induced by Gold Nanostar

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Article history: Received 18 Jan. 2024; Revised 1 Apr. 2024; Accepted 7 Apr. 2024; Published online 15 Dec. 2024

Abstract

Background: Polycystic ovary syndrome (PCOS) manifests as anovulation, oligomenorrhea, and hyperandrogenism in women of reproductive age, affecting around 5-10% of this population. There is a pressing want for a rapid, inexpensive, and uncomplicated approach to qualitatively and quantitatively detect free testosterone in patients with Polycystic Ovary Syndrome (PCOS).
Materials and methods: This study utilized surface-enhanced Raman spectroscopy (SERS) coupled with gold nano star (GNS) to amplify the optical signal to detect serum-free testosterone in PCOS patients. The study comprised 56 PCOS women age range (17-45years) who were sent from different governorates to the Baghdad Medical City.
Results: Highly resolved and high-quality surface-enhanced Raman scattering (SERS) spectra were obtained for free testosterone adsorbed on a substrate. These spectra exhibited characteristic bands of free testosterone and allowed for the detection of low levels (1.7 nM/mL), with an enhancement factor (EF) of 59 *105.
Conclusions: Based on the current knowledge this paper represents the pioneering effort to ascertain the concentration of free testosterone with the SERS technique. It is possible to efficiently early detection of PCOS by measuring the very low concentration of serum-free testosterone in a low-cost technique.

Keywords: SERS, GNS, EF, PCOS, Free Testosterone.

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disease in women, supplying them with several viable mixtures of symptoms and signs and a number of phenotypes. Women diagnosed with polycystic ovary syndrome (PCOS) face a heightened susceptibility to insulin resistance and hyperandrogenism, both of which can significantly impact their overall well-being around middle age and lead to enduring difficulties such as Irregular Menstrual Cycles, Infertility, Weight Management Issues, Excessive Hair Growth (Hirsutism, Acne and Skin Problems, Mood Disorders and Increased Risk of cancer

daises [1]. Testosterone & Free testosterone are the primary sex hormones and anabolic steroids found in males [2]. In both sexes, testosterone is an androstanoid with 17 beta-hydroxy and 3-oxo groups. It plays an essential role in health and well-being, affecting mood, behavior, and preventing osteoporosis [3]. Excessive androgen production is considered the primary factor driving the development of PCOS symptoms. Hyperandrogenism results from the overproduction of androgens by both the ovaries and the adrenals, leading to clinical manifestations such as hirsutism, acne, androgenic alopecia, and elevated testosterone levels [4]. Hyperandrogenism is a defining feature of PCOS in women, and it occurs due to the disruption of normal ovarian or adrenal function, resulting in excessive androgen production.

Androgen excess in PCOS initially impacts folliculogenesis, impairing it. During the early, gonadotropin-independent stage, increased androgen levels stimulate the formation of primordial follicles and raise the number of small antral follicles [5]. Numerous studies have indicated that elevated androgen levels pose a persistent risk factor in the onset of PCOS. Elevated testosterone levels are associated with obesity, particularly abdominal fat, insulin resistance, and an increased risk of glucose intolerance [6]. The latest clinical guidelines in endocrinology recommend utilizing elevated levels of total and free testosterone for the purpose of diagnosing PCOS [7]. Testosterone predominantly binds to sex hormone-binding globulin (SHBG) and albumin, with a little proportion (1-2%) circulating as unbound free testosterone (free T) not associated to proteins. Only the percentage of testosterone that is not bound to proteins can enter the cell and have an effect on androgen activity [8]. Free testosterone is suggested as the best responsive indicator for identifying androgen excess in medical practices [9].

In current years, bio-photonic and electrochemical detection techniques have been utilized in early clinical analysis. Specificity for any given biomarker is often realized using antibodies. The exact binding of an antibody to its target antigen in a multipart combination such as serum and plasma affords the revealing and quantification of diseases at levels as low as pictograms (pg) [10]. Sample concentration is the main issue criticized in bio-sensing. While ordinary sensors can handle concentrations of about little mM, nano-bio-sensors have the possibility to detect concentrations of about Femto Molar (fM) or even Ato Molar (aM). This ability to detect such fractional amounts of biomolecules stands as an advantage in detection diseases in the early stage of development so that specialists can interact and provide help to patients [11]. Bio-sensing has become increasingly popular due to the exceptional optical properties of NPs. Noble metal nanoparticles (NPs) have been utilized to establish a variety of highly sensitive bio-sensing techniques for nucleic acids, proteins, antibodies, enzymes, and other biological molecules. These techniques explore different physicochemical properties of NPs, including Localized surface Plasmon's Resonance (LSPR), fluorescence enhancement/quenching, SERS, electrochemical activity, and more [12]. Many research groups have reported success in detecting biomarkers or analytes using SERS systems; their ultimate goal is to advance POC technology.

Targeting cancers, diagnosing malaria, and recognizing bacterial meningitis are just some of the various uses for SERS techniques [13]. Gold nanostars (GNS) are a type of nanomaterial with many branches and sharp edges that behave as SERS "hotspots". These nanostars have been found to provide better SERS enhancement factors (EF) compared to nanospheres, making them the preferred choice for colloidal SERS substrates. SERS is an extension of standard Raman spectroscopy that uses an extremely weak optical effect to amplify Raman intensities by a factor of 104-106 in order to improve signal throughput and sensitivity. Because of its sensitivity and specificity, SERS technology has received a lot of attention and has been widely used in chemistry and biochemistry during the last few decades [14]. Different structures have been recognized for improving and evaluating SERS signals, as the metal's shape affects the organization of nanomaterials in the local electric field, and therefore, the distribution of SERS performance. GNS with different divisions and cutting edges function as SERS hotspots across diverse shapes, leading to higher SERS enhancement factors (EF) in comparison to nanorods and nanoparticles [15].

This study employed the SERS approach for the detection of Free Testosterone in the serum samples of Iraqi patients with PCOs. Subsequently, gold nanostructured SERS biosensors were utilized to evaluate their efficiency. To the best of our understanding, there has been no prior global research that has using the SERS approach to examine these predictive markers for PCOs.

2. Experimentally Procedures

The study comprised of 56 PCOS women and 27 control healthy women age range (17-45 years) who were sent from different governorates to Baghdad Medical City.
The levels of free testosterone in the serum were examined following the withdrawal of 5 ml of venous blood.

2.1 Chemicals and Instruments

A free testosterone kit was purchased from DRG Instrument GmbH /Germany) in different concentrations (0, 0.2,1,4,20and 100pg/ml). Nanoparticles, Spain, who supplied gold nano star (GNS) 0.2 mmol/L of 0.040 mg/mL of 40 nm of GNS form of polyvinyl pyrolidone.

2.2 Free testosterone Level measurement by Uv-Vis spectroscopy

The UV-Vis. analyses of samples (solutions) are carried out using UV- Vis. Shimadzu Spectrophotometer (UV-1800) to obtain the sample spectra.

2.3 Free testosterone Level measurement by SERS

In order to measure the SERS spectra 10µml of free testosterone with different concentration (0, 0.2,1,4,20and 100pg/ml) were mixed with one drop of 40nm colloidal gold nano star and mixed well at room temperature. In quartz cuvette, the mixture was exposed to 532nm for 10sec.

2.4 Statistical analysis

Frequency, percentage, mean, and standard deviation were utilized to depict the information in whole statistical analyses using SPSS version 25.0 software. When comparing more than two variables, an ANOVA is utilized to assess how the mean level of the numerical data differs. The correlation between numerical data was assessed using the Pearson correlation regression coefficient (r). A significant threshold of $p < 0.05$ was selected.

3. Results

3.1 Free testosterone Level measurement by Uv-Vis spectroscopy

In order to study the optical properties of free testosterone uv-vis spectrum as shown in Figure 1. The wide absorption spectrum was detected at regions from 200nm to 300nm. The absorption peaks increased with increasing in free testosterone concentrations.

3.2 Free testosterone Level measurement by SERS

From the Raman spectra, it may be inferred that only a subset of the Raman bands is now identified, specifically at 263, 662, 1469, 2154, 2672, 3288, and 4298 cm-1. Figure 2 demonstrates the presence of typical Raman bands for free testosterone in the range of approximately 662 to 1469 cm−1. These bands can be attributed to vibrations in the hydrocarbon, carbonyl, and carboxylic functional groups [13].

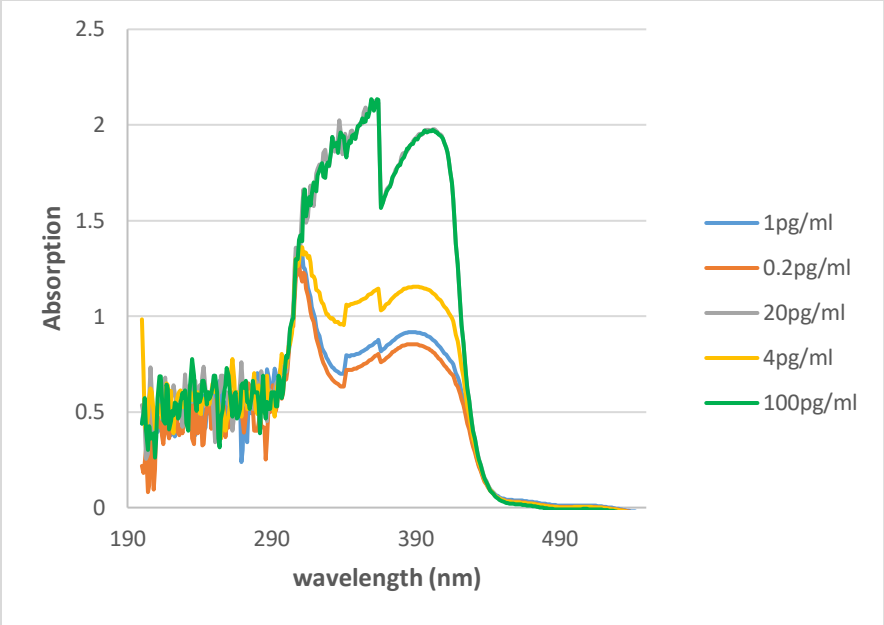

Fig. 1: Uv-Vis Spectrum of different concentrations of free Testosterone (0.2,1,4,20and 100pg/ml).

The SERS of free Testosterone 40 nm and GNS determined was found to have the most effective enhancement effect compared to other SERS substrates tested in this study. Therefore, GNS was selected as the preferred substrate for detecting trace amounts of free testosterone in blood. Highly resolved and high-quality surface-enhanced Raman scattering (SERS) spectra were obtained for free testosterone adsorbed on a substrate. These spectra exhibited characteristic bands of free testosterone and allowed for the detection of low levels (1.7 nM/mL). The SERS spectra were superior to the typical Raman spectrum of free testosterone. The relative strength variations observed in the characteristic bands were attributed to the interaction between free testosterone molecules and the active metal surface [16]. Therefore, it can be deduced that the limit of detection (LOD) for free testosterone is the same as the LOD for its most intense Raman peak, which is situated at a Raman shift of 1630 cm^{-1}. To estimate the concentration of free testosterone, we can utilize a univariate analysis that examines the strength of the free testosterone peak at 1640 cm^{-1}. This peak is associated with the stretching of the (C-C) and (C-N) rings. The Raman peak at 1640 cm^{-1}, which was excited at a wavelength of 532 nm, exhibited the maximum intensity among the Raman peaks associated with free testosterone. In comparison to other Raman peaks observed in samples that have been diluted of free testosterone, this particular peak was more easily quantifiable. Consequently, using this peak as our primary indicator resulted in a significantly reduced limit of detection for free testosterone. SERS largely arises from the electromagnetic enhancements of plasmonic nanostructures and, to a lesser extent, from chemical enhancement. The cutting tips on GNS possess structural properties that generate a highly confined electromagnetic field, making them 'hot spots' for SERS.

Prior research has indicated that GNS exhibited a notably greater enhancement in SERS in both colloids, when compared to gold nanorods or gold nanospheres, following their adsorption on a paper substrate [12]. The concentration of free testosterone was determined by utilizing the EF to assess the associated enhancement in performance for the paper substrate. This was accomplished at the aforementioned ideal conditions, as given by the subsequent mathematical expression[12]:

$$EF = (I_{SERS}/C_{SERS}) / (I_{RS}/C_{RS}) \quad (1)$$

Where:

I_{Raman} is the Raman signal intensities of free testosterone only.

I_{SERS} is the Raman signal intensity of free testosterone after adding GNSs.

C_{SERS}: sample concentration
C_{RS}: standard concentration

SERS and Raman measurements quantify the concentration of free testosterone compounds, while I_{SERS} and I_{RS} represent the intensity levels of free testosterone at 1640 cm^{-1} in SERS and classic Raman spectra. Similarly, C_{SERS} and C_{RS}. With the use of GNS, a mean EF of 59 * 105 was reached for the free testosterone under these optimum conditions.
Significantly, the degree of the SERS reaction was highest for the gold nanostars (GNS), as shown in Figure 2. The core principle of SERS is based on amplifying the spatial extent of metallic nano-materials to harness the excitation of SPR. However, aggregation was inadequately regulated, leading to heightened unpredictability and intricacy within the already complex system. To improve the topical electromagnetic field linked to the SPR, a viable strategy is to amplify topical nanomaterial curves.
It has been proved that when two spherical nanomaterials are properly clustered or clumped together, the SPR band is separated into different pieces, including longitudinal and transverse components. With the exception of two modes (quadrupole and dipole) in nanoparticles, there was no differentiation between them.

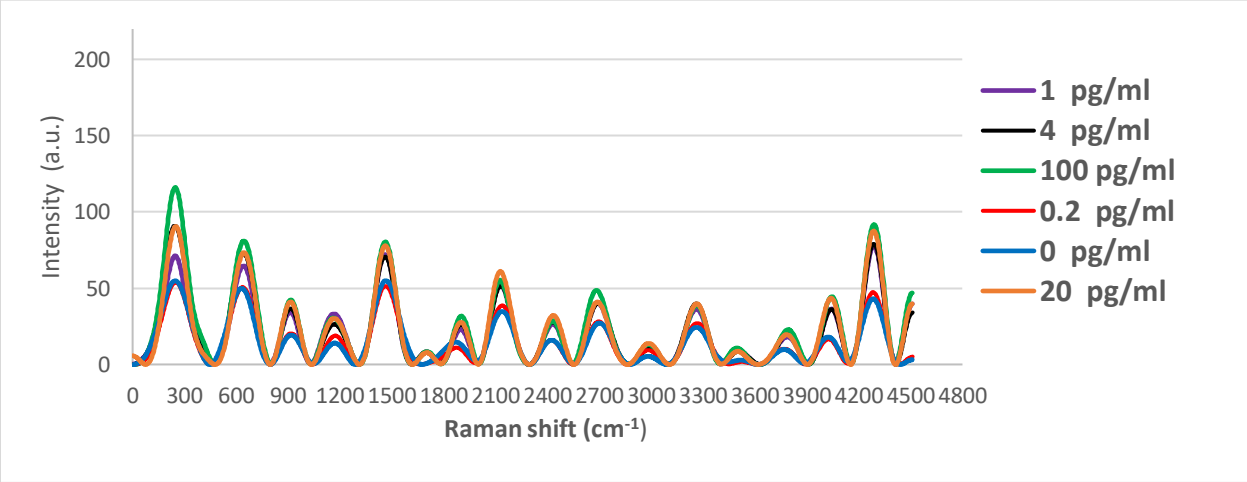

Fig. 2: Raman Spectrum of different concentrations of free Testosterone (0, 0.2,1,4,20and 100pg/ml) without GNS.

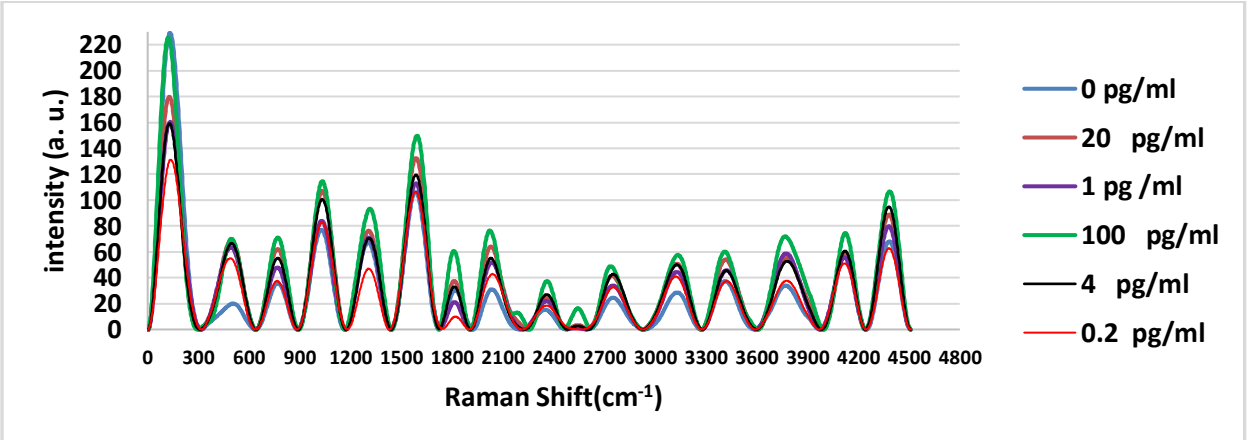

Fig. 3: Raman Spectrum of different concentrations of free Testosterone(0, 0.2,1,4,20and 100pg/ml) with GNS.

During the investigation of nano-triangles, researchers have observed four distinct surface resonances as a result of their anisotropic shape: out-of-plane dipole and quadrupole, as well as in-plane dipole/quadrupole. Nano stars possess a greater number of sharp edges and exhibit unique properties as highly complex an isotopically shaped nanoparticle. These nanoparticles exhibit many modes of vibration, each occurring at slightly different frequencies within gold materials. The Raman enhancement ratio was investigated as shown in figure 4.

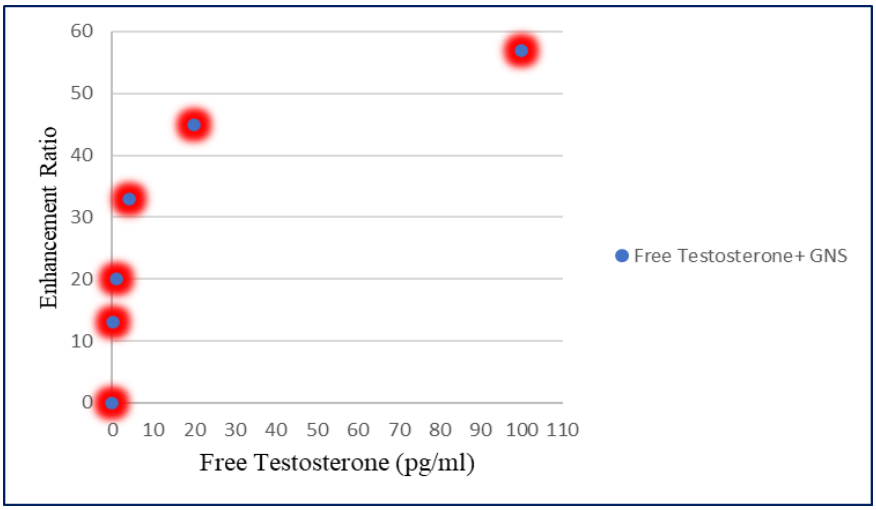

Fig. 4: Raman enhancement ratio of free testosterone with GNS (40 nm).

It has been proved that when two spherical nanomaterials are properly clustered or clumped together, the SPR band is separated into different pieces, including longitudinal and transverse components. With the exception of two modes (quadrupole and dipole) in nanoparticles, there was no differentiation between them. Nano stars possess a greater number of sharp edges and exhibit unique properties as highly complex isotopically shaped nanoparticles. These nanoparticles exhibit many modes of vibration, each occurring at slightly different frequencies within gold materials.

In order to assess the diagnostic efficacy of three different approaches (Elisa, Raman, SERS with GNSs) in detecting free testosterone at an early stage. These methods exhibit a much higher Area under the curve (AUC) compared to Raman pure. Among these methods, the one utilizing SER) with GNSs demonstrates the highest performance, with AUC values of 0.74, 0.79, and 0.88, respectively. AUC values closer to 1 indicate the screening measure reliably distinguishes among samples with patients and healthy women.

The comparison study was conducted to address the limitations of specificity and sensitivity associated with individual hormone visualization. Therefore, we employed gold nanostructures to improve the performance of surface-enhanced Raman spectroscopy (SERS). The concentration of free testosterone in PCOS patients was markedly decreased compared to the healthy control submit oneself ($P = 0.022$), As shown in Table 1.

4. Conclusions

In conclusion, the Free Testosterone SERS tags have been effectively synthesized and demonstrate that the amalgamation of GNSs can function as a sensor for free testosterone concentrations. This method was very simple and straight forward methods without any complications in procedures. The whole procedures done in five minutes only. The arrangement of the GNS produces organize an array (regular surface structure) that influences the SERS of the sample, resulting in increased EF which can be used to detect very low concentrations of free testosterone in a low-cost technique.

Table 1: Mean distribution of Free testosterone in sera of studied groups.

Assay	Age groups / Year	PCOS			Controls		
		N	Mean	Std. Deviation	N	Mean	Std. Deviation
Free Testosterone by traditional Method (Elisa)	15 - 25	20	25.7033	15.58490	11	58.0705	46.49889
	26 - 35	26	38.3068	25.01109	10	46.2768	20.65601
	36 - 45	10	30.5996	9.03356	6	48.9217	50.61042
	P- value	P = 0.108 NS			P = 0.783 NS		
Free Testosterone by SERS	15 - 25	20	1.11075	.662984	11	.54700	.325418
	26 - 35	26	.80550	.571512	10	.52070	.187116
	36 - 45	10	.50240	.210335	6	.53083	.209523
	P- value	P = 0.022 S			27	P = 0.973 NS	

5. Institutional Review Board (IRB) license

This study has received approval from the Ethical Committee of the Ministry of Health (MOH) of Iraq as cross-sectional research. Each participant granted their informed consent.
Approval from the Scientific Research Ethics Committee was received (No. 31) in 10/1/2024.

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تشخيص متلازمة المبيض المتعدد الكيسات باستخدام مستويات هرمون التستوستيرون الحرة عبر تحليل رامان الطيفي المحسن للسطح بواسطة الذهب النانومتري ذو الشكل النجمي

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الخلاصة

الخلفية: تظهر متلازمة المبيض المتعدد الكيسات (PCOS) على شكل انقطاع الإباضة، وندرة الطمث، وفرط الأندروجين لدى النساء في سن الإنجاب، مما يؤثر على حوالي 5-10٪ من هذه الفئة من السكان .هناك حاجة ملحة لاتباع نهج سريع وغير مكلف وغير معقد للكشف نوعيًا وكميًا عن هرمون التستوستيرون الحر لدى المرضى الذين يعانون من متلازمة المبيض المتعدد الكيسات.(PCOS)

المواد والطرق: استخدمت هذه الدراسة مطيافية رامان المحسّنة على السطح (SERS) مقترنة بنجمة النانو الذهبية (GNS) لتضخيم الإشارة الضوئية للكشف عن هرمون التستوستيرون الخالي من المصل لدى مرضى متلازمة تكيس المبايض .شملت الدراسة 56 امرأة مصابة بمتلازمة تكيس المبايض من الفئة العمرية (17-45 سنة) تم إرسالهن من محافظات مختلفة إلى مدينة الطب في بغداد.

النتائج: تم الحصول على أطياف تشتت رامان (SERS) عالية الدقة وعالية الجودة من أجل هرمون التستوستيرون الحر الممتز على الركيزة .أظهرت هذه الأطياف نطاقات مميزة من هرمون التستوستيرون الحر وسمحت باكتشاف المستويات المنخفضة (1.7 نانومتر/مل)، مع عامل تعزيز (EF) قدره 59 * 105.

الاستنتاجات: استنادا إلى المعرفة الحالية تمثل هذه الورقة الجهد الرائد للتأكد من تركيز هرمون التستوستيرون الحر باستخدام تقنية SERS. من الممكن الكشف المبكر عن متلازمة تكيس المبايض بكفاءة عن طريق قياس التركيز المنخفض للغاية لهرمون التستوستيرون الخالي من المصل باستخدام تقنية منخفضة التكلفة.

الكلمات المفتاحية : مطيافية رامان السطحية المحسنة, الذهب النانومتري ذو الشكل النجمي, عامل التعزيز, متلازمة المبيض المتعدد الكيسات, هرمون التستوستيرون الحر.