

Bio-hybrid materials for immunoassay-based sensing of cortisol

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Abstract Sol–gel encapsulation has been used as the basis for detecting cortisol by an immunoassay approach. Previous research showed that antibodies immobilized in the pores of a sol–gel derived silica were able to bind cortisol and be used as an immunosensor. However, this approach was not effective when measuring cortisol levels in human serum because of interference from other fluorescence sources. The present paper describes a protocol which overcomes these limitations and enables sol–gel immunoassays to effectively measure cortisol in human serum over the physiological range of cortisol blood concentrations in an adult (2–28 µg/dL). The method involves a standard additions approach in which various amounts of cortisol are added to the serum. The cortisol concentration values obtained with our sol–gel immunoassay were typically within 10% of the values obtained by traditional analytical methods. The protocol presented here represents

a significant contribution to sol–gel sensing and immunoassays in particular, because of the ability to detect an analyte in human serum. In addition, this work reports the first comparison between results from a sol–gel immunosensor and an alternative immuno-binding method for analyte detection.

Keywords Sol–gel · Biosensing · Immunoassay · Cortisol · Standard additions

1 Introduction

The sol–gel process is a low-temperature method for synthesizing inorganic glass at room temperature. The chemistry is based on the hydrolysis and condensation of molecular precursors such as metal alkoxides [1–4]. The sol, a stable suspension of colloidal solid particles in a liquid, undergoes gelation which leads to the formation of a porous 3-dimensional interconnected matrix with an interstitial liquid phase [5]. The sol–gel process has been the subject of numerous studies and the ability to fabricate various oxides into optically transparent monoliths, films, as well as fibers and opaque powders, is well established [5–9].

During the 1990s researchers realized that sol–gel methods could be used to immobilize a wide range of enzymes and other proteins, leading to materials which exhibit biochemical responses [10–15]. As long as the biomolecule was soluble in the aqueous or alcohol-based solvent, it could be incorporated within the pores of the inorganic matrix, and effectively impart its properties to the resulting solid [1, 6, 9]. Among the different biomolecule immobilization methods, sol–gel encapsulation has emerged as one of the most attractive approaches. This

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route has demonstrated a number of advantages including the preservation of the structure and activity of the biomolecule, prevention of self-aggregation, protection from outside environmental conditions, improved thermal resistance and storage capability, good mechanical durability, and good optical properties [14, 16–19]. The latter is of central importance because of the ability to use the optical properties of the biomolecule for a variety of sensor applications. Another key feature associated with sol–gel based materials is the microstructure of the matrix. The intrinsic pore size can be tailored such that small analytes can diffuse in and bind or react with the encapsulated biomolecule while the larger, encapsulated biomolecule is unable to diffuse out of the matrix [1, 17]. It is significant to mention that the field of sol–gel immobilization continues to be extremely active. The sensor area has grown considerably through the design of sol–gel materials with enhanced biorecognition, while the ability to encapsulate membrane-bound proteins has led to applications in high throughput drug screening [20, 21].

This paper presents work in another important area, that of using sol–gel encapsulation as the basis for immunoassays. Immunoassays are common techniques for measuring a wide variety of analytes in standard solutions and in serum samples. Over the past decade, sol–gel encapsulation of biomolecules for immunoassays has generated considerable interest because the encapsulation overcomes some limitations in the technology [17, 22–25]. A problematic issue for traditional immunoassays, such as enzyme-linked immunosorbent assays (ELISA), is that certain biomolecules, such as proteins, lose their stability or functionality upon immobilization [17]. Common immobilization techniques, such as physical adsorption and covalent bonding [17] have well known limitations. Physical adsorption of the biomolecule to the surface utilizes weak bonding. In contrast, covalent bonding provides a strong attachment of the biomolecule to the surface but difficulties arise in isolating the biomolecule for analysis and detection. In the present paper, we used sol–gel encapsulation to not only achieve long-term storage but also to make the analytical protocol compatible with on-orbit detection.

The immunoassay of interest in this paper is the hormone cortisol. Cortisol is a glucocorticoid that regulates metabolism and provides resistance to stress in the body [26]. The physiological range of cortisol blood concentrations in an adult is 2–28 $\mu\text{g}/\text{dL}$. High levels of cortisol indicate a high amount of stress and lead to a significant reduction in the immune response. For this reason, cortisol is of interest to NASA because it can serve as a biomarker for the health of the astronaut crew. On-orbit monitoring is particularly important for prolonged space missions as the microgravity environment of space places additional stress, physically and psychologically, on humans during these missions [27].

Traditional methods developed for the detection of biomolecules such as cortisol involve radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) [28–31]. However, these methods are not viable for use on-orbit because of concerns over radioactivity (RIA) and multiple liquid handling steps (ELISA). As a result, the measurement of hormones and other biomolecules occurs on earth, i.e., only before and after space flights.

The present paper builds upon our prior work in which we demonstrated a sol–gel immunoassay for cortisol. In that work, we showed that antibodies immobilized in the pores of the sol–gel derived silica were able to bind cortisol. We also demonstrated that the sol–gel immunoassay was able to measure cortisol over a physiologically relevant range and that only one washing was required in the analysis. This last point is of considerable importance for on-orbit applications. However, when we went to use this approach to measure cortisol levels in human serum, we experienced a number of difficulties related to background fluorescence from the presence of other biomolecules in serum as well as non-specific binding. We overcame these problems by developing a measurement protocol based on the “standard additions” technique. Our experimental results are in excellent agreement with measurements made by a standard immunoassay method. The results reported here are among the first sol–gel immunoassays to be compared directly to standard analytical methods and represent the first report of using a sol–gel based assay method to monitor an analyte present in human serum.

2 Background

Sol–gel immunoassays for cortisol were used to measure cortisol in the physiological relevant range of 1–100 $\mu\text{g dL}^{-1}$. A competitive immunoassay approach was used based on doping anti-cortisol in a silica sol in which the resulting sol–gel matrix was designed to have an average pore diameter of approximately the same size as the antibody. Figure 1 shows typical calibration curves from these sol–gel immunoassays [22]. The fluorescence decrease with the logarithmic increase in unlabeled cortisol is expected for competitive immunoassays [32–34]. Figure 1a presents the experimentally obtained fluorescence data while in Fig. 1b the fluorescence intensity has been normalized according to the standard approach for analyzing competitive immunoassays. In addition to the monolithic samples shown here, competitive immunoassay experiments were also carried out using sol–gel thin films which immobilized anti-cortisol. These materials exhibited an analogous response to that shown in Fig. 1. An advantage of using thin films is that it reduces the required assay time substantially.

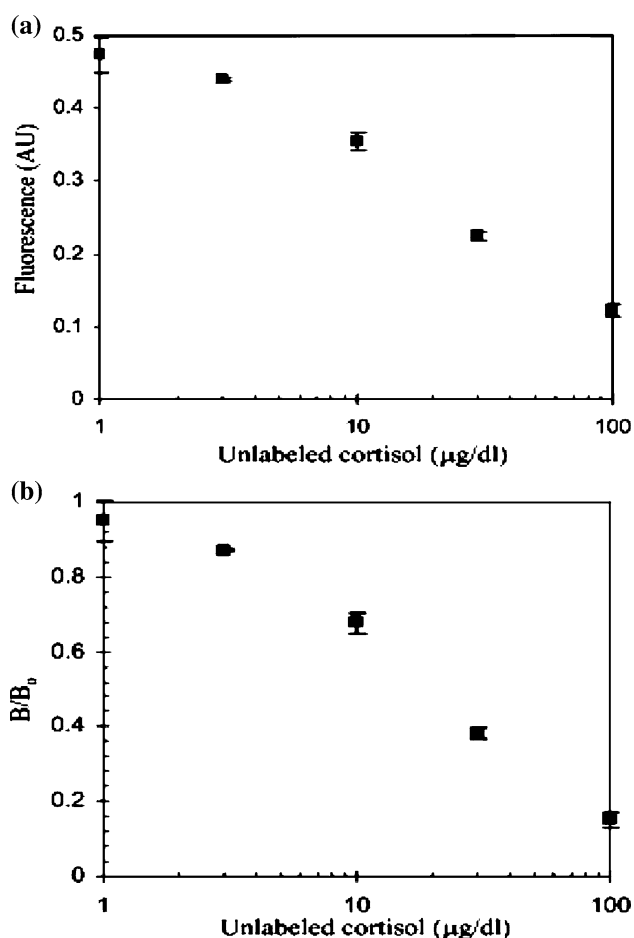


Fig. 1 **a** Fluorescence signal versus the logarithm of the unlabeled cortisol concentration for sol-gel competitive immunoassays using silica monoliths containing 0.16 μM anti-cortisol antibody. **b** Fluorescence is converted to normalized intensity B/B_0 (reprinted with permission from Zhou et al [22])

Unfortunately, sol-gel competitive immunoassays were unable to detect cortisol levels accurately when human serum samples were used. Figure 2 compares sol-gel immunoassay results obtained from standard cortisol solutions with results obtained for a few serum samples. The latter were supplied by the NASA Johnson Space Center (NASA-JSC). There is a significant discrepancy between the cortisol concentrations from NASA-JSC, analyzed by standard immunoassay methods, and those obtained from our sol-gel immunoassays (Table 1). We hypothesize that luminescence from other biomolecules present in serum may interfere with the fluorescence signal coming from the labeled cortisol used in the sol-gel immunoassays. The background intensity may come from the luminescence of other biomolecules that could covalently bind to the hydrophilic silica matrix. Serum contains intrinsic fluors, or biomolecules that are naturally fluorescent, such as proteins containing amino acids with phenyl rings [35]. The emission from these fluors could interfere

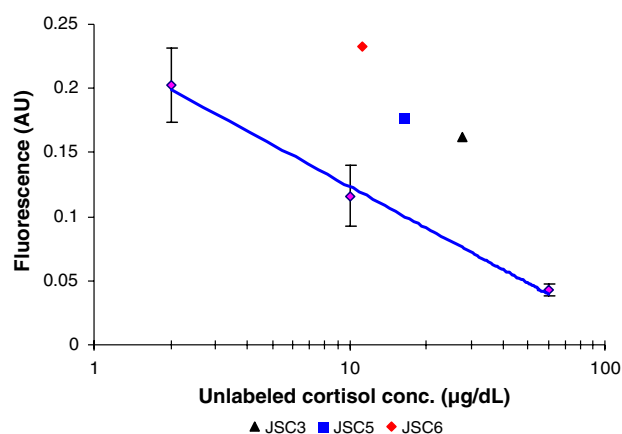


Fig. 2 Fluorescence versus unlabeled cortisol concentration for standard cortisol solutions (calibration curve) and some serum samples (JSC3, JSC5, JSC6). The unlabeled cortisol concentrations for the serum samples are values obtained from NASA-JSC

Table 1 Comparison of cortisol concentration values reported by NASA-JSC with the values we calculated from calibration curves of standard cortisol solutions

Serum sample	NASA-JSC ($\mu\text{g dL}^{-1}$)	Calculated from the sol-gel immunoassay ($\mu\text{g dL}^{-1}$)
JSC3	27.7	4.4 ± 1.7
JSC5	16.3	3.2 ± 0.5
JSC6	11.2	1.0 ± 0.2

with that coming from the labeled cortisol, thus masking the signal. To resolve these problems, we developed a protocol which enabled us to obtain accurate detection of cortisol levels in human serum.

3 Experimental details

3.1 Materials

Tetramethylorthosilicate (TMOS) and hydrocortisone (cortisol) were purchased from Sigma-Aldrich Chemical Co. Phosphate buffer saline solution (1X PBS), sodium phosphate buffer, methanol, and hydrochloric acid were obtained from Fisher Scientific. Monoclonal cortisol antibody (Lot #L28, density = 2.14 mg/mL) was purchased from East Coast Biologics. Human serum samples were supplied by the NASA-JSC. Oregon Green conjugated cortisol (OG-cortisol) was purchased from Molecular Probes, Inc.

Fluorescence measurements were made with a Fluoroskan Ascent Type 374 fluorimeter from ThermoLabsystems.

A Dayton DC Speed Control was used to control the velocity of the dip-coating machine. The Immulite 2000 analyzer from Diagnostic Products Corporation was used by NASA-JSC to determine the cortisol concentration levels of the serum samples.

3.2 Preparation of antibody doped sol-gel thin films

The sol was prepared by combining 4 mL of TMOS, 0.9 mL of DI water, and 0.2 mL of 0.02 N HCl and sonicating the solution for 30 min [22]. After sonication, methanol was added to the hydrolyzed sol and filtered with a 0.2 μm syringe filter [22]. Two hundred and thirty micro liters of the monoclonal antibody to cortisol (density = 2.14 mg/mL) was added to 7.15 mL of 5 mM sodium phosphate buffer solution with pH 6.0. The buffered antibody solution was then added to 5.85 mL of the sol/methanol mixture. After 15 min, thin films were dip-coated on to clean glass cover slips. The thin films on the cover slips were then allowed to dry in air for 100 s before being stored in 1X PBS in a refrigerator for later use.

3.3 Competitive immunoassays for cortisol

Figure 3 illustrates the specific details of the approach used for sol-gel competitive immunoassays. In a typical competitive immunoassay, both fluorescently labeled cortisol (conjugated with Oregon Green) and unlabeled cortisol from serum bind competitively to the sol-gel encapsulated antibody [36]. Fluorescence is detected from the labeled cortisol and can be correlated with the concentration of unlabeled cortisol from serum. A higher fluorescent signal is indicative of a lower analyte concentration since more of

the labeled analyte is bound to the antibody. For the cortisol immunoassay, an assay mixture of 1.2 mL of 1X PBS, 1.5 μL of 20 μM OG-cortisol, and 50 μL of the treated serum sample was added to a cuvette. The antibody-doped sol-gel thin films were then added to the cuvette and the assay mixture was incubated on a rotator for 20 min. The thin films were then rinsed with deionized water and placed over individual wells in a 96-well plate. Fluorescence measurements were taken in triplicate with the excitation and emission wavelengths being 485 and 538 nm, respectively.

4 Results and discussion

4.1 Standard additions procedure and analysis

The standard additions method has been widely used to detect a variety of analytes in complex media, such as biological fluids, where interference in analyte signal can cause inaccuracies [37–42]. The method used here is similar to the Gran plot standard additions method used previously to determine unknown parameters, such as the slope and y-intercept of a calibration curve for the detection and monitoring of ions [43–46]. The Gran plot standard addition technique also eliminates the need for reference calibration curves [46]. A diagram depicting the method of standard additions is shown in Fig. 4. A human serum sample of unknown cortisol concentration was divided equally into three tubes. Then, equal volumes of different cortisol concentrations were added to each tube. Treated serum samples #1, 2, and 3 all came from one subject and were of equal cortisol concentration.

Fig. 3 Procedure for sol-gel competitive immunoassays using both thin film and bulk monolithic materials

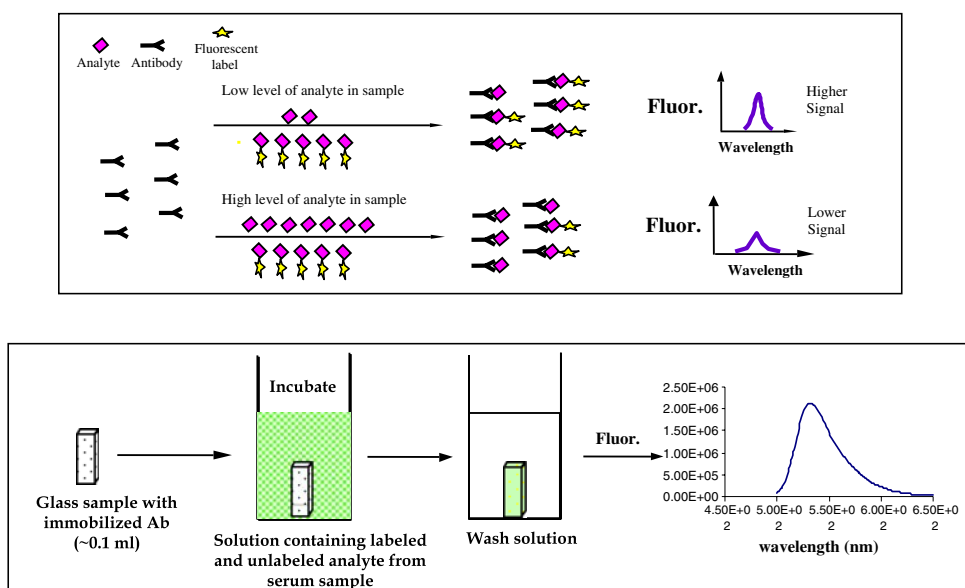
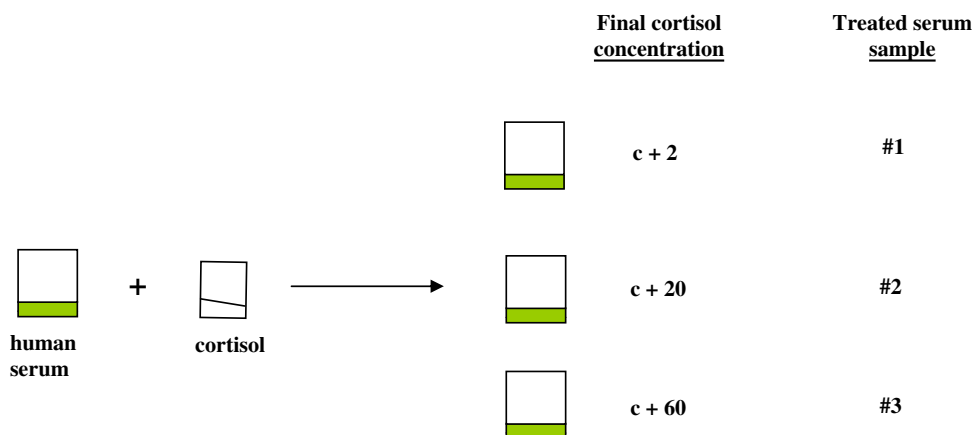


Fig. 4 Standard additions method for a human serum sample



The protocol involves taking 600 μL of a human serum sample of unknown cortisol concentration, c , and dividing it into three microcentrifuge tubes with 200 μL of serum in each tube. Then, 6.9 μL of 60 $\mu\text{g}/\text{dL}$ of cortisol was added to the first tube, 6.9 μL of 600 $\mu\text{g}/\text{dL}$ of cortisol was added to the second tube, and 6.9 μL of 1,800 $\mu\text{g}/\text{dL}$ of cortisol was added to the last tube. The volumes and concentrations for the added cortisol were calculated with the assumption that the serum sample contained no cortisol. A conventional method for representing data from competitive immunoassays is shown in Fig. 1, where the fluorescent intensity is plotted versus the logarithm of the unlabeled analyte concentration [32]. In our case, this would yield a linear relationship:

$$f = m * \ln(c) + b \tag{1}$$

where f is fluorescence intensity, c is the cortisol concentration, and m and b are the slope and y-intercept of the line, respectively. Three equations can be written for each of the three treated serum samples with different final cortisol concentrations:

$$f_1 = m * \ln(c + 2) + b \tag{2}$$

$$f_2 = m * \ln(c + 20) + b \tag{3}$$

$$f_3 = m * \ln(c + 60) + b \tag{4}$$

where f_1 , f_2 , and f_3 are the fluorescent intensities obtained from the competitive immunoassay on serum samples 1, 2, and 3, respectively. The three unknown variables c , m , and b can be determined by solving these three simultaneous Eqs. 2–4. To minimize error, the actual cortisol concentration is $1.03 * c$.

4.2 Competitive immunoassays on spiked human serum samples

The standard additions method enabled us to circumvent the fluorescence interference problem and obtain accurate

values for cortisol in human serum. The results from thin film sol-gel immunoassays carried out on seven different “spiked” human serum samples (a–g) are shown in Fig. 5, in which fluorescence is plotted as a function of unlabeled cortisol concentration. The linear relationship between

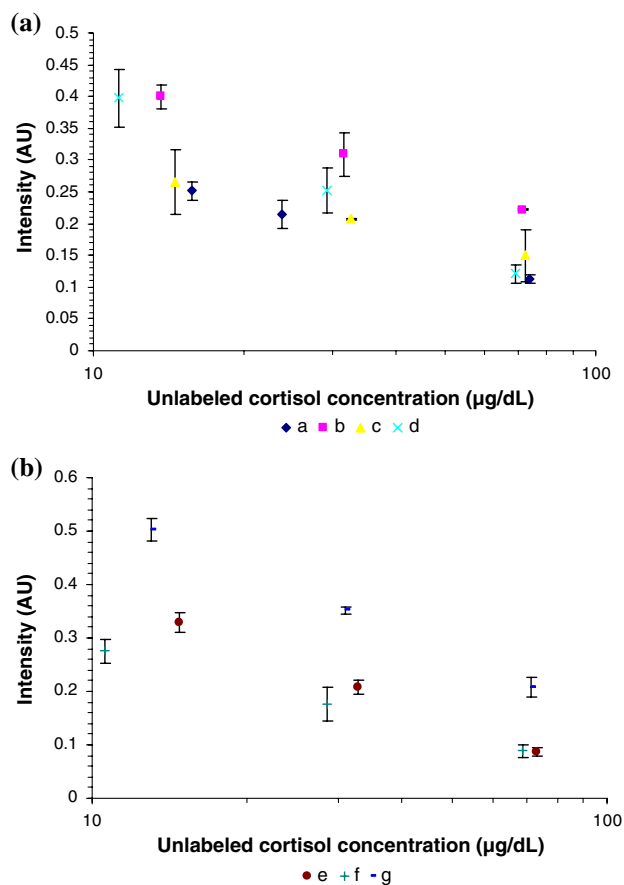


Fig. 5 Fluorescence versus the logarithm of the unlabeled cortisol concentration of seven “spiked” serum samples: **a** samples (a–d). **b** Samples (e–g). Error bars represent the standard deviations from the mean values for the data taken in triplicate

fluorescence and the logarithm of the cortisol concentration (Eq. 1) was confirmed for these “spiked” serum samples. The fit of the data was such that the fraction of variance (*R*-squared value) was equal to unity and linearity was maintained for all serum samples. These assays have good reproducibility as the average standard deviation in intensity for the seven “spiked” serum samples of Fig. 5 was 9.4%. This value is comparable to that of previous sol–gel thin film immunoassays for cortisol [22].

The analytical procedure outlined in Sect. 3.1 was performed on numerous human serum samples to determine their cortisol content. The calibration curve parameters *m* and *b* were determined from fitted linear calibration curves (Eq. 1) with *y* defined as $\exp(b/m)$. The values of these parameters (*y*, *m*, and *b*) for the seven samples of Fig. 5 are summarized in Table 2. The slopes of the linear curves (*m*) are not constant due to the varying intensities from the assay. The slopes and *y*-intercepts of these linear calibration curves have no apparent physical meaning as they are just variables used to obtain cortisol levels in the serum. Since *f*₁, *f*₂, *f*₃, *m*, and *b* are all known quantities, the cortisol concentration of each human serum sample was calculated by substituting these 5 parameters into Eqs. 2–4. Cortisol concentrations determined from our sol–gel immunosensor for serum samples a–g are shown in Table 2.

The cortisol concentration values obtained from our sol–gel immunosensor were compared with measurements made by NASA-JSC on the same samples. At NASA-JSC the instrument used to make cortisol measurements is based on a technique which involves immuno-binding, multiple washings of unbound material, and detection of a chemiluminescent substrate from the assay. The cortisol concentrations of the serum samples ranged from 4.3 to 17.9 µg/dL.

We are able to successfully correlate the cortisol concentration values of the sol–gel immunoassay to within 15% of the values obtained by NASA-JSC. Moreover, for

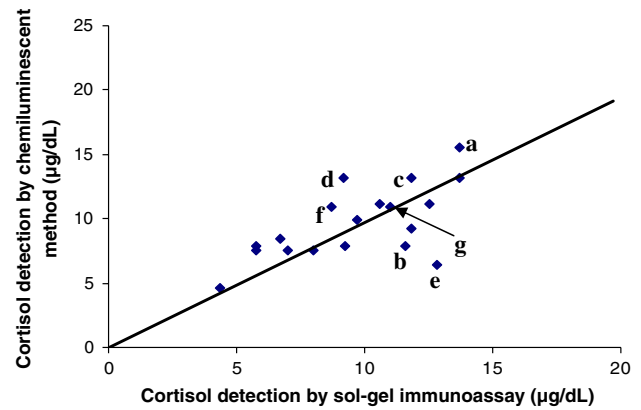


Fig. 6 Comparison of cortisol concentration values reported by NASA-JSC (chemiluminescent method) versus values obtained by the sol–gel immunoassay. The slope of the curve is unity. Results are shown for multiple human serum samples, including a–g as indicated

several samples, there was less than a 5% difference. As shown in Fig. 6, which directly compares the two approaches, there is very good correlation between the cortisol concentration values obtained from NASA-JSC and our sol–gel immunosensor. The data indicates that there were very few outliers such as sample e. Our results are comparable, if not better than previous cortisol sensing studies in serum in which fluorescence polarization immunoassays were correlated with radioimmunoassays [47, 48]. Table 3 shows the results of multiple tests on various serum samples using the standard additions method. For this set of samples, NASA-JSC had coefficient of variances ranging from 6.8 to 9.4% with the chemiluminescent method, comparable to the coefficient of variances obtained with our sol–gel immunosensor using the standard additions method (Table 3). A final point to mention is that the total time to perform this measurement procedure with the thin film immunoassay is approximately 30 min. These results show promise for adapting the standard additions method for a miniaturized sol–gel immunoassay system that can be used on-orbit.

Table 2 Calibration curve parameters (*y*, *m*, *b*) and cortisol concentrations for “spiked” serum samples a–g

Serum sample	<i>y</i>	<i>m</i>	<i>b</i>	Calculated cortisol conc. (µg/dL)
a	3.84e–03	–0.09	0.50	13.7
b	1.75e–03	–0.11	0.68	11.6
c	1.71e–03	–0.07	0.46	12.5
d	6.52e–03	–0.15	0.76	9.20
e	7.83e–03	–0.15	0.74	12.8
f	6.01e–03	–0.10	0.51	8.60
g	4.24e–03	–0.17	0.95	11.0

Table 3 Cortisol concentration values for various human serum samples determined by NASA-JSC and by the sol-gel immunosensor using the method of standard additions (standard deviations from mean shown). The number of times (*n*) an individual serum sample has been analyzed is also shown. Cortisol concentration (µg/dL)

NASA-JSC	Our sol–gel immunosensor	<i>n</i>
7.6	6.9 ± 1.1	3
7.84	8.9 ± 2.9	3
10.9	9.8 ± 1.6	2
11.1	11.6 ± 1.3	2
13.2	11.4 ± 1.9	4

5 Conclusions

A competitive immunoassay based on sol–gel encapsulation of antibodies was successfully used for sensing cortisol. By adopting a standard additions method for analysis, cortisol was accurately detected in human serum samples. The cortisol concentration values obtained with our sol–gel immunoassay were typically within 15% of the values obtained by traditional analytical methods. The protocol presented here represents a significant contribution to sol–gel sensing and immunoassays in particular, because of the ability to detect an analyte in human serum. In addition, this work reports the first comparison between results from a sol–gel immunosensor and an alternative immuno-binding method for analyte detection. These results are promising for developing a miniaturized sol–gel bioassay system for in-flight monitoring of cortisol and other important biomarkers.

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References

- Dunn B, Miller JM, Dave BC, Valentine JS, Zink JI (1998) *Acta Mater* 46:737. doi:10.1016/S1359-6454(97)00254-1
- Livage J, Beteille F, Roux C, Chatry M, Davidson P (1998) *Acta Mater* 46:743. doi:10.1016/S1359-6454(97)00255-3
- Livage J, Sanchez C, Henry M, Doeuff S (1989) *Solid State Ion* 32–33:633. doi:10.1016/0167-2738(89)90338-X
- Brinker CJ, Scherer GW (1989) *Sol-gel science: the physics and chemistry of sol-gel processing*. Academic Press, San Diego
- Klein L (1988) *Sol-gel technology for thin films, fibers, preforms, electronics, and specialty shapes*. Noyes Publications, New Jersey
- Ellerby LM, Nishida CR, Nishida F, Yamanaka SA, Dunn B, Valentine JS, Zink JI (1992) *Science* 255:1113. doi:10.1126/science.1312257
- Wang R, Narang U, Prasad PN, Bright FV (1993) *Anal Chem* 65:2671. doi:10.1021/ac00067a020
- Yamanaka SA, Nguyen NP, Dunn B, Valentine JS, Zink JI (1996) *J Sol-Gel Sci Technol* 7:117. doi:10.1007/BF00401891
- Avnir D (1995) *Acc Chem Res* 28:328. doi:10.1021/ar00056a002
- Avnir D, Braun S, Lev O, Ottolenghi M (1994) *Chem Mater* 6:1605. doi:10.1021/cm00046a008
- Braun S, Rappoport S, Zusman R, Avnir D, Ottolenghi M (1990) *Mater Lett* 10:1. doi:10.1016/0167-577X(90)90002-4
- Yamanaka SA, Nishida F, Ellerby LM, Nishida CR, Dunn B, Valentine JS, Zink JI (1992) *Chem Mater* 4:495. doi:10.1021/cm00021a001
- Zink JI, Yamanaka SA, Ellerby LM, Valentine JS, Nishida F, Dunn B (1994) *J Sol-Gel Sci Technol* 2:791. doi:10.1007/BF00486352
- Avnir D, Coradin T, Lev O, Livage J (2006) *J Mater Chem* 16:1013. doi:10.1039/b512706h
- Miller JM, Dunn B, Valentine JS, Zink JI (1996) *J Non-Cryst Solids* 202:279. doi:10.1016/0022-3093(96)00191-3
- Dave BC, Miller JM, Dunn B, Valentine JS, Zink JI (1997) *J Sol-Gel Sci Technol* 8:629
- Dave BC, Dunn B, Valentine JS, Zink JI (1994) *Anal Chem* 66:1127A. doi:10.1021/ac00094a001
- Lan EH, Dave BC, Fukuto JM, Dunn B, Zink JI, Valentine JS (1999) *J Mater Chem* 9:45. doi:10.1039/a805541f
- Nguyen DT, Smit M, Dunn B, Zink JI (2002) *Chem Mater* 14:4300. doi:10.1021/cm020398t
- Holthoff EL, Bright FV (2007) *Acc Chem Res* 40:756. doi:10.1021/ar700087t
- Brennan JD (2007) *Acc Chem Res* 40:827. doi:10.1021/ar6000268
- Zhou JC, Chuang MH, Lan EH, Dunn B, Gillman PL, Smith SM (2004) *J Mater Chem* 14:2311. doi:10.1039/b401902d
- Lan EH, Dunn B, Zink JI (2000) *Chem Mater* 12:1874. doi:10.1021/cm990726y
- Li CI, Lin YH, Shih CL, Tsaur JP, Chau LK (2002) *Biosens Bioelectron* 17:323. doi:10.1016/S0956-5663(01)00287-1
- Doong RA, Tsai HC (2001) *Anal Chim Acta* 434:239. doi:10.1016/S0003-2670(01)00853-4
- Tortora G, Grabowski SR (2003) *Principles of anatomy & physiology*. John Wiley & Sons, New York
- Borchers AT, Keen CL, Gershwin ME (2002) *Nutr* 18:889. doi:10.1016/S0899-9007(02)00913-9
- Berson SA, Yalow RS (1968) *Clin Chim Acta* 22:51. doi:10.1016/0009-8981(68)90247-7
- Foster LB, Dunn RT (1974) *Clin Chem* 20:365
- Kobayashi Y, Tsubota N, Miyai K, Watanabe F (1980) *Steroids* 36:178
- Lewis JG, Manley L, Whitlow JC, Elder PA (1992) *Steroids* 57:82. doi:10.1016/0039-128X(92)90034-7
- Harris DC (2003) *Quantitative chemical analysis*. W.H. Freeman, New York
- Pesce AJ, Kaplan LA (1987) *Methods in clinical chemistry*. C.V. Mosby Company, St. Louis, MO
- Ashkar FS (1983) *Radiobioassay*. CRC Press, Boca Raton, FL
- Boyer RF (1993) *Modern experimental biochemistry*. Addison-Wesley Publishing Company, Massachusetts
- Masseyeff RF, Albert WH, Staines NA (1993) *Methods of immunological analysis, vol 1*. VCH Publishers Inc., New York, NY, VCH Verlagsgesellschaft mbH, Weinheim (Federal Republic of Germany)
- Hund E, Massart DL, Smeyers-Verbeke J (1999) *J Pharm Biomed Anal* 21:23. doi:10.1016/S0731-7085(99)00111-9
- Kim S, Kim HJ (2005) *Bioresour Technol* 96:1457. doi:10.1016/j.biortech.2004.12.003
- Manivannan A, Ramakrishnan L, Seehra MS, Granite E, Butler JE, Tryk DA, Fujishima A (2005) *J Electroanal Chem* 577:287. doi:10.1016/j.jelechem.2004.12.006
- Basilicata P, Miraglia N, Pieri M, Acampora A, Soleo L, Sannolo N (2005) *J Chromatogr B Analyt Technol Biomed Life Sci* 818:293. doi:10.1016/j.jchromb.2005.01.013
- Yang CC, Kumar AS, Zen JM (2005) *Anal Biochem* 338:278. doi:10.1016/j.ab.2004.12.015

42. Fleet B, Ho AYW (1973) *Talanta* 20:793. doi:[10.1016/0039-9140\(73\)80195-X](https://doi.org/10.1016/0039-9140(73)80195-X)
43. Li H (1991) *Anal Lett* 24:473
44. Martyak NM (1992) *Plat Surf Finish* 79:60
45. Araujo AN, Montenegro MCBSM, Kousalova L, Sklenarova H, Solich P, Olmos RP (2004) *Anal Chim Acta* 505:161. doi:[10.1016/S0003-2670\(03\)00096-5](https://doi.org/10.1016/S0003-2670(03)00096-5)
46. Yperman J, Backer AD, Vos A, Franco D, Mullens J, Van Poucke LC (1993) *Anal Chim Acta* 273:511. doi:[10.1016/0003-2670\(93\)80197-S](https://doi.org/10.1016/0003-2670(93)80197-S)
47. Kobayashi Y, Tsubota N, Miyai K, Watanabe F (1979) *Steroids* 34:829. doi:[10.1016/0039-128X\(79\)90095-3](https://doi.org/10.1016/0039-128X(79)90095-3)
48. Kumar S, Safa AM, Deodhar SD (1976) *Clin Chem* 22:1845