Talanta 81 (2010) 1703–1710

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A chemometric approach for optimizing protein covalent immobilization on magnetic core–shell nanoparticles in view of an alternative immunoassay

Bruno Teste[a], Jérôme Vial[a], Stéphanie Descroix[a,*], Thomas Georgelin[b], Jean-Michel Siaugue[b], Jan Petr[c], Anne Varenne[c], Marie-Claire Hennion[a]

[a] Physicochimie des Electrolytes, Colloïdes et Sciences Analytiques (PECSA) UMR 7195, CNRS-ESPCI-UPMC, 10 rue Vauquelin, 75231 Paris Cedex 05, France
[b] Physicochimie des Electrolytes, Colloïdes et Sciences Analytiques (PECSA) UMR 7195 CNRS-UPMC-ESPCI, 4 place Jussieu, 75252 Paris Cedex 05, France
[c] Physicochimie des Electrolytes, Colloïdes et Sciences Analytiques (PECSA) UMR 7195 CNRS-ENSCP-UMPC, 11 rue Pierre et Marie Curie, 75231 Paris Cedex 05, France

ARTICLE INFO

Article history:
Received 7 October 2009
Received in revised form 10 March 2010
Accepted 18 March 2010
Available online 25 March 2010

Keywords:
Covalent immobilization
α-Lactalbumin
Magnetic core–shell nanoparticles
Experimental design
EDC
NHS

ABSTRACT

A chemometric approach was developed to optimize the grafting of a bovine milk allergen: α-Lactalbumin (α-Lac) on colloidal functionalized magnetic core–shell nanoparticles (MCSNP). Such nanoparticles, functionalized with polyethyleneglycol and amino groups, exhibit a 30 nm physical diameter and behave as a quasi-homogeneous system. The α-Lac immobilization was achieved through the covalent binding between MCSNP amino groups and α-Lac carboxylic moieties using the well-known tandem carbodiimide (EDC) and hydroxysulfosuccinimide (NHS). In this study, a chemometric approach was employed to highlight the parameters influencing the number of grafted proteins on the MCSNP. Three factors were evaluated: the ratio in concentration between EDC and α-Lac, between NHS and EDC and the concentration of α-Lac. After a first full factorial design to delimit the region of the space where the optimum could be located, a central composite design was then carried out to predict the best grafting conditions. It was established and experimentally confirmed that the optimum parameters are [EDC]/[α-Lac] = 25; [NHS]/[EDC] = 1.55 and α-Lac = 24.85 nmol mL^{-1}. In these optimal conditions, MCSNP surface was successfully saturated with α-Lac (34 α-Lac/MCSNP) with a high reproducibility (RSD = 2%). The colloidal stability of MCSNP grafted with α-Lac as well as the immunological interactions using anti α-Lac antibody were then investigated in different buffers. The results emphasized that a 50 mM MES buffer (pH 6) allows an efficient immune capture and a satisfying colloidal stability which provide an immunological interaction in homogeneous liquid phase.

1. Introduction

In the field of bioanalytical systems, the role played by biomolecules immobilization can be considered as a crucial point since it conditions the maintaining of good catalytic activity [1] and protein stability [2]. Immunochemical techniques, consisting in capturing and quantifying antigens (Ag) or antibodies (Ab), are widely used because of their high specificity and sensitivity. Among the variety of immunoassays, enzyme linked immunosorbent assay (ELISA) offers the skills previously mentioned in an easy process. The first step of immunoassays consists in an efficient Ag or Ab immobilization according to the employed format. Two main ways exist to achieve biomolecules grafting, namely the covalent binding and the physical adsorption.

In the case of ELISA, biomolecules grafting is usually performed by physical adsorption. This simple process suffers from major drawbacks like molecules attachment which occurs randomly, proteins denaturation, decrease of immunological capture efficiency and problems of molecules accessibility, notably in reason of overlapping phenomenon [3]. Covalent anchoring ensures a stronger and more reproducible attachment compared to randomized adsorption. Many cross-linkers such as glutaraldehyde [4], epoxy [5] or carbodiimide [6] are widely employed for protein or DNA grafting in a two steps procedure: surface activation and biomolecules immobilization. Some grafting procedure using biotinylation permitted molecules orientation increasing immunological capture efficiency [7].

Another important point is that ELISA techniques cannot pretend to reflect homogeneous liquid phase *in vivo* immune interactions as one of the partner remains immobilized on solid support. Moreover, since only one partner of the immunological interaction is really diffusing in solution, it results in longer analysis time. In order to overcome these problems, the use of latex beads [8] and magnetic beads [9–12] as biomolecule support inside microsystems allows a better accessibility as well as an increase of surface to volume ratio and a reduced diffusion distance. Nevertheless,

* Corresponding author. Tel.: +33 1 40 79 46 44; fax: +33 1 40 79 46 54.
E-mail address: stephanie.descroix@espci.fr (S. Descroix).

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.03.027

these microsystems analyses are still performed in heterogeneous phase since the beads are of micrometric size and cannot diffuse in solution.

Nanoparticles are getting an interesting tool for various biological technologies. They were mostly used in label technologies for rapid and ultra sensitive detection [13,14] but rarely as an immunological capture support. Li and coworkers [4] successfully constructed piezoelectric immunosensor based on magnetic nanoparticles attached to quartz crystal, but such immunoassay was still performed in heterogeneous phase.

We have previously designed magnetic core–shell nanoparticles (MCSNP) [15] functionalized both with polyethyleneglycol (PEG) which allows a reduction of biomolecules non-specific adsorption and amino groups which permit biomolecules covalent immobilization. These MCSNP were characterized by different techniques and notably by capillary electrophoresis in a previous work [16]. Noteworthy, these MCSNP present an interesting colloidal stability for months without forming cluster, and should then constitute good candidates for an immunological capture support in homogeneous phase. Another feature of the MCSNP is based on their magnetic properties that allow their trapping when a magnetic field is applied.

The challenge of our project is to develop an immunoassay that should provide analysis time reduction, high sensitivity and should mimic homogeneous liquid phase *in vivo* immune interactions. This last point remains crucial in Ag–Ab affinity evaluation as a characterization tool for diseases evolution. The feasibility of this innovative immunological support will be demonstrated in the case of milk allergy diagnosis using α-Lac.

The first step of this work consists in optimized Ag covalent immobilization on MCSNP while keeping their colloidal stability in view of performing immunoassay in homogeneous phase. Examples of proteins grafting on micrometric beads have already been published but, up to our knowledge, no study concerning optimization of protein immobilization on sub-100 nm MCSNP dedicated for immunoassay has ever been reported.

This article describes the grafting optimization of α-Lac at the surface of these sub-100 nm MCSNP. It is based on a one step amide bond formation between MCSNP amino groups and α-Lac carboxylic moieties using EDC and NHS [17] under neutral pH conditions. Classical way for optimization consists in varying only one factor at a time (univariate approach) [6]. This optimization approach would require a lot of runs with a lack of efficiency and could fail to carry out the true optimum because it neglects factors interactions, i.e. coupled effects of different factors. The design of experiment (multivariate approach) [18] enables studying effects of all factors and interactions by applying simultaneous controlled variations. In the present study, grafting optimization was performed thanks to this chemometric approach. A two steps strategy was implemented to first define the region of the experimental domain where the optimum could be located using a two levels factorial design, and then to predict the α-Lac optimum grafting conditions using a central composite design. Finally, the influence of storage conditions (buffer, pH) in sight of immobilized α-Lac biorecognition and α-Lac grafted MCSNP stability was evaluated.

2. Materials and methods

2.1. Materials

N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC); N-Hydroxysulfosuccinimide sodium salt (NHS); 3-(N-Morpholino)propanesulfonic acid sodium salt (MOPS); 2-(N-Morpholino)ethanesulfonic acid sodium salt (MES); 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES); tetraethoxyorthosilicate (TEOS); 3-(aminopropyl)triethoxysilane (APTS); α-Lactalbumin from bovine milk (α-Lac); *O*-Phenylenediamine (OPD); Tween® 20; formic acid and acetic acid were purchased from Sigma–Aldrich (St-Quentin Fallavier, France). Citric acid was purchased from Merck (Nogent sur Marne, France). 2-[Methoxy(polyethyleneoxy)propyl]-trimethoxysilane (PEOS), containing 3-6 ethylene oxide groups, was purchased from Gelest (Morrisville, PA, USA). Polyclonal IgG anti α-Lactalbumin antibody horseradish peroxidase (HRP) conjugated from goats (specific Ab) was purchased from GeneTex® Inc (Irvine, CA, USA). Polyclonal anti IgG HRP conjugated from goats (non-specific Ab) was purchased from Biosys Technology (Paris, France). 30% H_2O_2, 2N HCl and 2N NaOH solutions were obtained from VWR (Strasbourg, France). Microplates 96 wells were purchased from Greiner BioOne (Courtaboeuf, France). 2 mL Eppendorf tube were obtained from Eppendorf AG (Hamburg, Germany). Micro BCA protein assay was purchased from Pierce (Rockford, IL, USA).

2.2. Apparatus and softwares

All rinsing steps were performed using sigma 2K15 centrifuges from Meditech Scientific (Clamart, France) or MS column with MACS separator magnet from Miltenyi (Paris, France). MCSNP diameter measurements were performed with Nano ZS Zetasizer (Malvern Instrument, Worcestershire, UK). Ultrasonic wave cleaner 45 Hz was obtained from VWR (Strasbourg, France). Solutions absorbance was read using SpectraMaxM2 microplate reader from Molecular Devices (St Grégoire, France). Grafting solutions were shaken using rotary disk agitator SB3 from VWR (Strasbourg, France).

The statistical data analyses were performed using JMP 5.1 (S.A.S Institute Inc, Cary, NC, USA) and Excel 2003 (Microsoft Corporation, Courtaboeuf, France).

2.3. Magnetic core–shell nanoparticles synthesis

Briefly, maghemite nanoparticles (7 nm in mean physical diameter) have been prepared by co-precipitation of Fe^{2+} and Fe^{3+} ions under alkaline conditions as described by Massart [19]. Nanoparticles were then coated by citrate anions and dispersed in water [20]. These maghemite nanoparticles were further encapsulated in silica shells. A first silica shell was prepared in ethanolic medium in the presence of ammonia as a catalyst by condensation of TEOS [21]. The silica shell functionalization was carried out through a second step by simultaneous condensation of a silica amine derivated compound: APTS and a silica polyethyleneglycol derivated compound: PEOS. The concurrent addition of a small amount of TEOS resulted in the formation of a crosslinked silica shell [15]. The surface particle charge density can be tuned by varying the APTS to PEOS molar ratios, the amount of PEOS staying constant while the quantity of APTS increasing. The ratio used for our study was a 1:1 APTS/PEOS ratio. The reaction of silica condensation is carried out over a night and particles suspension was destabilized by diethyl ether. A red precipitate was formed and separated by magnetic settling. The precipitate was twice washed with a mixture of diethyl ether and ethanol (15:1) and then redispersed in 10 mM MOPS buffer (pH 7.5). This solution of amino-PEG functionalized MCSNP (approximately 3.1×10^{14} MCSNP mL^{-1}) was stable for months.

2.4. Preparation of grafting solution

350 μL of MCSNP solution (3.1×10^{14} MCSNP mL^{-1}) were mixed with 200 μL of EDC (various concentrations), 200 μL of NHS (various concentrations) and 400 μL of α-Lac solution (various concentrations) in a 2 mL Eppendorf tube. Each reagent solution was

freshly prepared in 10 mM MOPS/NaOH buffer (pH 7.5). The concentrations of EDC, NHS and α-Lac solutions are given in Section 3. All tubes were shaken by rotary movement (15 rpm) at 20 °C for 15 h.

2.5. Magnetic core–shell nanoparticles washing

2.5.1. Washing steps using magnetical properties

First, MS column was washed 3 times with 500 μL 50 mM MOPS/NaOH buffer (pH 7.5). Then, the column was inserted inside MACS separator magnet. 400 μL of the MCSNP solution (1×10^{14} MCSNP mL^{-1}) just after Ag grafting or after interaction with Ab, were injected into MS column using micropipette. The trapped MCSNP were washed 3 times with 400 μL 50 mM MOPS/NaOH buffer (pH 7.5). The column was then removed from the magnet and the MCSNP were eluted with 400 μL 50 mM MOPS/NaOH buffer (pH 7.5).

2.5.2. Washing steps using centrifugation

400 μL from MCSNP solution (1×10^{14} MCSNP mL^{-1}) just after Ag grafting or after interaction with Ab, were centrifuged at 14,000 × *g* during 10 min using a 2 mL Eppendorf tube. The supernatant was discarded and MCSNP were resolubilized in 400 μL 50 mM MOPS/NaOH buffer (pH 7.5) using an ultrasonic wave apparatus for 5 min to facilitate MCSNP solubilization. This step was repeated 3 times. MCSNP were finally solubilized in 400 μL 50 mM MOPS/NaOH buffer (pH 7.5).

2.6. Quantitation of grafted α-Lac on magnetic core–shell nanoparticles

Quantitation of grafted α-Lac on MCSNP (MCSNPα-Lac) was performed thanks to BCA protein assay test kit (according manufacturer recommendation). 150 μL from the rinsed MCSNPα-Lac solution (1×10^{14} MCSNP mL^{-1}) were mixed with 150 μL of BCA test work reagent inside a microplate well at 37 °C. After 2 h incubation, MCSNPα-Lac were discarded using centrifugation or MS column in order to avoid any scattering effect and the absorbance of 300 μL supernatant solution was read at 570 nm.

2.7. Immobilized α-Lac/antibody interaction and detection

400 μL from the rinsed MCSNPα-Lac solution (1×10^{14} MCSNPα-Lac mL^{-1}) were mixed with 50 μL 90 ng mL^{-1} specific or non-specific HRP labelled Ab (10 ng mL^{-1} final concentration) for 2 h at room temperature, then rinsed 3 times with a 50 mM PBS-Tween (0.1%, v/v) and resolubilized in 400 μL 50 mM MOPS/NaOH (pH 7.5). 100 μL from this solution were mixed with 100 μL 100 mM citrate buffer solution containing OPD (1 mg mL^{-1}) and H_2O_2 (0.06%, w/v) for Ab detection. After 30 min, the Ab conjugated HRP catalytic reaction was stopped with 50 μL 2 M HCl. MCSNP were discarded using centrifugation or MS column in order to avoid any scattering effect and the absorbance of 150 μL supernatant solution was read at 490 nm.

3. Designs of experiments

3.1. Choice of the responses

To evaluate the performance of the immobilization procedure, we chose as relevant response the amount of grafted α-Lac on each MCSNP. Although it is not directly involved as a factor of experimental design, MCSNP hydrodynamic diameter after grafting was also measured using Dynamic Light Scattering method. Indeed, an increase of MCSNP hydrodynamic diameter would indicate the formation of clusters due to covalent binding between α-Lac and several MCSNP. As the hydrodynamic diameter of bared MCSNP was 65 nm (results not shown), we considered that hydrodynamic diameter higher than 130 nm would indicate cluster formation. The results obtained have shown that no cluster formation occurred as the hydrodynamic diameter of grafted MCSNP with α-Lac was 79 ± 6 nm. In the following discussion, the amount of immobilized α-Lac was the only response taken into account.

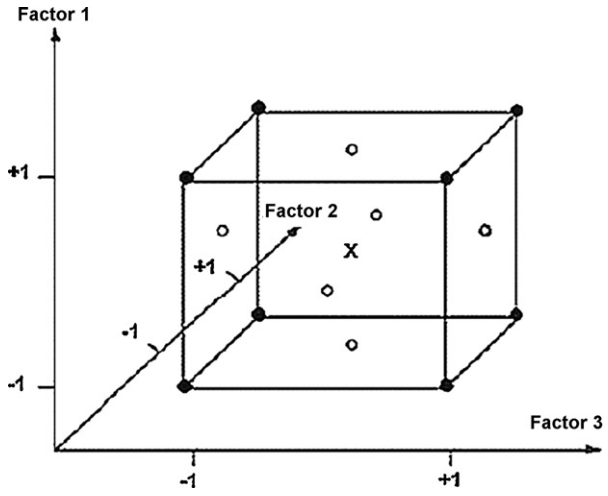

Fig. 1. 3D representation of a central composite design composed of 8 points from factorial design (●), one central point (×) (4 repetitions) and 6 axial points (○).

3.2. Choice of the factors

Preliminary experiments (data not shown) concerning immobilization protocol using EDC/NHS couple led us to select 3 factors:

1. EDC/α-Lac concentration ratio.
2. NHS/EDC concentration ratio.
3. α-Lac molar concentration.

3.3. Choice of the experimental designs

The optimization strategy was sequential and proceeded in two steps. First we elaborated a two-level full factorial design (2^3) for 3 factors. It allowed calculating the effect of each factor, to get a first outline of the response variations according to factors levels and to determine the experimental domain where the optimum could be located. A central composite design (CCD) [22] consisting of a second 2^3 factorial design, one central (4 repetitions) and 6 axial points, was then built. This CCD permits quadratic modelling useful for determining optimal grafting conditions. Fig. 1 shows a representation of the CCD in the experimental space. The 6 axial points are located in the centre of each cube faces.

3.4. Factors levels

[EDC]/[α-Lac], [NHS]/[EDC], and [α-Lac] were respectively denoted *X*1, *X*2 and *X*3 where *X* = (factor value − factor values mean)/(range/2), the high level being +1, the low level being −1 and the medium level being 0. Factors levels, in initial and coded values of the factorial design are shown in Table 1, they were chosen to cover the widest experimental but non-denaturing conditions. Indeed, we observed that beyond high values of factors ([EDC]/[α-Lac] > 100 and [α-Lac] > 31.05 nmol mL^{-1}), proteins precipitation and MCSNP aggregation were favored. Thus, the domain of study was built far enough from these limit values in order to avoid these phenomenon. MCSNP concentration was constant

Table 1
Domain of study for the 2^3 factorial design and the central composite design.

	1st factorial design					Central composite design			
	Factors	High level (+1)	Low level (−1)	Medium level (0)		Factors	High level (+1)	Low level (−1)	Medium level (0)
X1	[EDC]/[α-Lac]	25	2	13.5	*X1*	[EDC]/[α-Lac]	25	13.5	19.25
X2	[NHS]/[EDC]	2	0	1	*X2*	[NHS]/[EDC]	2	1	1.5
X3	α-Lac (μg) (nmol mL^{-1})	400	100	250	*X3*	α-Lac (μg) (nmol mL^{-1})	400	250	325
		24.85	7.14	17.86			24.85	17.86	23.21

(3.1×10^{14} MCSNP mL^{-1}). The quantity of α-Lac was chosen to be in excess compared to MCSNP, approximately from 40- to 175-fold higher.

The domain of the CCD, defined from the results of the first 2^3 design was a narrower area of the previous domain and is given in Table 1.

3.5. Matrix of experiments

The 1st factorial design was composed of 12 experiments: 8 for the factorial design and 4 repetitions of the central point.

The central composite design was composed of 18 experiments: 8 for the second factorial design, 6 axial points and 4 repetitions for the central point (Table 2). All the experiments were randomized to avoid being affected by uncontrolled factors variations.

3.6. Model construction and coefficients calculation for the 1st factorial design

The following model has been used for responses modelling:

$$\hat{Y}_{p1} = \text{intercept} + E1 \cdot X1 + E2 \cdot X2 + E3 \cdot X3 + E12 \cdot X1X2 + E13 \cdot X1X3 + E23 \cdot X2X3 + E123 \cdot X1X2X3$$

where $\hat{Y}_{p1}$ is the predicted response from linear model, intercept was the constant term, *Ei* the coefficient (effect) of the factor *Xi*, *Eij* the coefficient of the interaction *XiXj* between factors *i* and *j*, and *Eijk* the coefficient of the interaction *XiXjXk* between the three factors.

Coefficients calculations were made thanks to multiple linear regression [23] of the 8 experiments from 1st factorial design using excel software.

Table 2
Matrix of experiments for the 1st factorial design and for the central composite design.

Experiments	1st factorial design			Experiments	Central composite design		
	X1	*X2*	*X3*		*X1*	*X2*	*X3*
1	1	1	1	1	1	1	1
2	1	1	−1	2	1	1	−1
3	1	−1	1	3	1	−1	1
4	1	−1	−1	4	1	−1	−1
5	−1	1	1	5	−1	1	1
6	−1	1	−1	6	−1	1	−1
7	−1	−1	1	7	−1	−1	1
8	−1	−1	−1	8	−1	−1	−1
9	0	0	0	9	0	0	0
10	0	0	0	10	0	0	0
11	0	0	0	11	0	0	0
12	0	0	0	12	0	0	0
				13	0	0	−1
				14	0	−1	0
				15	0	1	0
				16	1	0	0
				17	0	0	1
				18	−1	0	0

3.7. Significant factors of the 1st factorial design

Factors significance was evaluated thanks to a Student's test (t) [24] which consists in comparing the coefficient previously definite to its relative standard deviation (RSD) value. First, we estimated the response RSD, denoted $\hat{\sigma}$, using central point repetitions; we supposed that the RSD was constant on the entire experimental domain. The observed t value (t_{obs1}) for each factors and interactions from 1st factorial design was calculated with the following equation:

$$t_{obs1} = \frac{\text{coefficient}}{\hat{\sigma}/\sqrt{N}}$$

where N is the number of experiments (=2^3).

The t_{obs1} was compared to a tabulated reference (t_{ref}) [24] depending on the first kind risk (α) and the number of degrees of freedom (dof) [25].

The decision rules were as follows: if $t_{obs1} \in [-t_{ref}(\alpha, \text{dof}); t_{ref}(\alpha, \text{dof})]$ then the coefficient was not significant, if not the coefficient was significant.

3.8. Model validity for the 1st factorial design

Validation of the linear model consisted in comparing predicted and experimental values of the response at the central point thanks to a t-test (t_{obs2}). The null hypothesis (Ho) was as follows: the central point predicted value was equal to the central point experimental value. The observed value for t_{obs2} was:

$$t_{obs2} = \frac{|Y_e - Y_p|}{\hat{\sigma}\sqrt{(1/n_e) + (1/N)}}$$

where Y_e is the experimental response observed at the central point, Y_p the predicted response at the central point, n_e (=4) the number of repetitions at the central point and N the number of experiments (=2^3).

The decision rules were as follows: if $t_{obs2} \in [-t_{ref}(\alpha, \text{dof}); t_{ref}(\alpha, \text{dof})]$ then Ho is accepted, if not Ho is rejected.

3.9. Model construction and coefficients calculation for central composite design

The following quadratic model has been used for responses modelling:

$$\hat{Y}_{p2} = \text{intercept} + E1 \cdot X1 + E2 \cdot X2 + E3 \cdot X3 + E12 \cdot X1X2 + E13 \cdot X1X3 + E23 \cdot X2X3 + E123 \cdot X1X2X3 + E11 \cdot X1X1 + E22 \cdot X2X2 + E33 \cdot X3X3$$

where $\hat{Y}_{p2}$ is the predicted response of the quadratic model, intercept the constant term, *Ei* the coefficient of the factor *Xi*, *Eij* the coefficient of the interaction *XiXj* between factors *i* and *j*, *Eijk* the coefficient of the interaction *XiXjXk* between the three factors and *EiEi* the coefficient of the square term *XiXi*.

Table 3
Estimation of the coefficients and t_{obs1} calculation for each factor and interaction from 1st factorial design.

Experiments	Mean	*X*1	*X*2	*X*3	*X*1*X*2	*X*1*X*3	*X*2*X*3	*X*1*X*2*X*3	*Y*
1	1	1	1	1	1	1	1	1	33.5
2	1	1	1	−1	1	−1	−1	−1	8.1
3	1	1	−1	1	−1	1	−1	−1	22.4
4	1	1	−1	−1	−1	−1	1	1	13.8
5	1	−1	1	1	−1	−1	1	−1	1.7
6	1	−1	1	−1	−1	1	−1	1	0
7	1	−1	−1	1	1	−1	−1	1	1
8	1	−1	−1	−1	1	1	1	−1	0
Coefficients	9.75	9.25	0.75	4.5	0.75	4	2	2	
t_{obs1}	25.9	24.6	2	12	2	10.6	5.3	5.3	
t_{ref} (5%, 3 dof)	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	

Multiple linear regressions from the 18 experimental values of the CCD permitted to calculate model coefficients using JMP 5.1 software.

3.10. Significant terms for central composite design

Coefficients significance has been tested using Student's test which consisted in comparing the coefficient value to its RSD stemming from regression residual RSD. If the *P* value [24] (given by JMP 5.1 software) was lower than the first kind risk then the factor was considered as significant. These tests were carried out for information purposes only. All terms, even non-significant coefficients, were kept for the modelling used to determine the optimum.

3.11. Model validation for central composite design

The validation of the quadratic model was carried out by comparing the predicted response and the experimental response observed in predicted optimal conditions thanks to a *t*-test (t_{obs3}).

$$t_{obs3} = \frac{\left|Y_{op} - Y_{oe}\right|}{\sqrt{\hat{\sigma}_p^2 + (\hat{\sigma}_e^2/n_e)}}$$

where Y_{oe} is the optimal experimental response, Y_{op} the optimal predicted response, $\hat{\sigma}_p$ the quadratic model predicted residual RSD in the optimum conditions, $\hat{\sigma}_e$ the Y_{oe} RSD, n_e (=4) the number of repetitions for Y_{oe}.

Rules decisions have already been reported for linear model adequacy.

4. Results

4.1. Results of the 1st factorial design

The results of coefficients estimations and t_{obs1} from the 1st factorial design are reported in Table 3.

t_{obs1} and t_{ref} comparison underlined *X*1, *X*3, *X*1*X*3, *X*2*X*3 and *X*1*X*2*X*3 significant influence on the response in the 1st factorial design domain. For *X*1 = −1 (Table 3: entry 5, 6, 7, 8), the low value of the ratio [EDC]/[α-Lac] leads to no grafting of the protein on the MCSNP surface. The presence of polymer chains at the surface of the MCSNP is responsible for problems of anchoring sites accessibility, namely the amino groups. For those reasons, numerous carboxylic groups have to be activated in one protein in order to obtain an efficient grafting process at the surface of the MCSNP. In the same way, at low concentration of protein (*X*3 = −1, Table 3: entry 2, 4), poor covering of the surface is obtained. It means that high activation of proteins is not sufficient, notably because once activated, carboxylic groups can react with amino groups of the same or others proteins. So in order to have sufficient reactions with the few accessible MCSNP amino groups, the concentration of protein has to be high. The last factor seems to have less influence (Table 3: entry 3): it means that in our conditions, the activated ester due to the reaction between one carboxylic group and one EDC is reactive enough and that no supplementary activation with NHS is really necessary to have an efficient reaction with an amino group. We equally observed that high values of the response were obtained for high factors values (Table 3: entry 1).

The comparison of predicted *Y* (=9.8) and experimental *Y* (=21.9) at the central point revealed that the two values were statistically different with t_{obs2} (=39.5) > t_{ref} (=3.2). This means that the linear model was not satisfactory enough for modelling response vari-

Table 4
Results of the central composite design.

Experiments	Mean	*X*1	*X*2	*X*3	*X*1*X*2	*X*1*X*3	*X*2*X*3	*X*1*X*2*X*3	*X*1*X*1	*X*2*X*2	*X*3*X*3	*Y*
1	1	1	1	1	1	1	1	1	1	1	1	32.5
2	1	1	1	−1	1	−1	−1	−1	1	1	1	21.8
3	1	1	−1	1	−1	1	−1	−1	1	1	1	32.1
4	1	1	−1	−1	−1	−1	1	1	1	1	1	17.7
5	1	−1	1	1	−1	−1	1	−1	1	1	1	28.1
6	1	−1	1	−1	−1	1	−1	1	1	1	1	15.9
7	1	−1	−1	1	1	−1	−1	1	1	1	1	24.6
8	1	−1	−1	−1	1	1	1	−1	1	1	1	22.0
9	1	0	0	0	0	0	0	0	0	0	0	26.1
10	1	0	0	0	0	0	0	0	0	0	0	24.8
11	1	0	0	0	0	0	0	0	0	0	0	25.7
12	1	0	0	0	0	0	0	0	0	0	0	25.4
13	1	0	0	−1	0	0	0	0	0	0	1	21.5
14	1	0	−1	0	0	0	0	0	0	1	0	25.6
15	1	0	1	0	0	0	0	0	0	1	0	26.9
16	1	1	0	0	0	0	0	0	1	0	0	28.9
17	1	0	0	1	0	0	0	0	0	0	1	36.0
18	1	−1	0	0	0	0	0	0	1	0	0	25.0

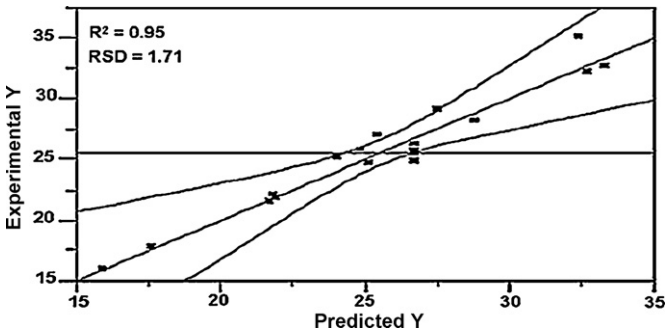

Fig. 2. Correlation graph between experimental *Y* and predicted *Y* from central composite design. The dots represent the experimental points, the regression line (centre line) is surrounded by the confidence curves underlining quadratic model residual RSD.

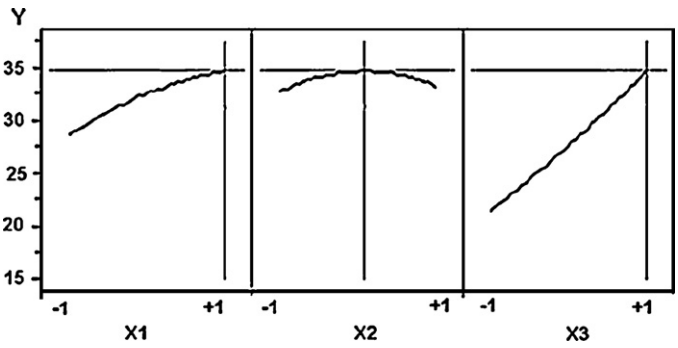

Fig. 3. Response variations according to the three main factors. Intersection indicates the factor coordinate for the highest response value.

ations according to the studied factors. So, both to answer this matter and to have the ability to carry out a true optimization, a second experimental design was build in a narrower domain, defined thanks to first factorial design results. This new experimental domain should more likely contain the optimum. Moreover this second design must be compatible with optimization constrains, i.e. it must be a response surface design.

4.2. Results for the central composite design

The experimental results for the CCD are exposed in Table 4.

Fig. 2 presents the experimental response versus the predicted one. Homogeneous scattering of residuals, R^2 value of 0.95 and acceptable residual RSD of 1.71 indicate the global appropriateness of the quadratic model proposed. The confidence curves in Fig. 2 are not containing the horizontal line thus underlining the quadratic model significance.

Quadratic model factors and interactions coefficients estimations were gathered in Table 5.

A factor is considered as significant when P value $< \alpha$. When $\alpha = 5\%$, $X1$ and $X3$ had a significant effect on the response. The positive value of $X1$ and $X3$ coefficient indicated that the response increased when the factors varied from their low to their high levels. $X2$ does not seem to have any influence on the response when $X1$ and $X3$ were put in their high and middle levels whereas it was significant when $X1$ and $X3$ were put in their low levels as shown in Table 5: these results underlined $X1X2X3$ significance. When $\alpha = 10\%$, $X1$, $X3$, $X1X2X3$ and $X1X3$ had a significant influence on the response.

The significance of [EDC]/[α-Lac] ($X1$) as well as [α-Lac] ($X3$) was expected. As shown before, the covalent grafting of α-Lac is possible because of the EDC ester formation on α-Lac carboxylic moieties in order to create an amide bond with the accessible MCSNP amino groups thus the value of the ratio [EDC]/[α-Lac] is a crucial point. Another part of the problem is to favor the reaction between proteins and nanoparticles, leading to high values of the protein concentration, in order to efficiently compete with the numerous side reactions between activated carboxylic group and amino groups of proteins. The EDC ester is able to perform the amide reaction, so the replacement of EDC ester to NHS ester is not necessary in our context thus explaining the non-significance of [NHS]/[EDC] factor ($X2$).

Fig. 3 represents the variations of the response as a function of each factor. It indicated that the response increased monotonously with $X1$ in a parabolic profile and increased linearly with $X3$. The variation as a function of $X2$ is parabolic: it increased till reaching a maximum, then decreased. The presence of NHS allows a slight increase of the number of grafted proteins till reach a maximum. When the quantity of NHS is too important, the increasing activation of carboxylic acid groups leads to more side reactions between proteins thus favoring proteins clusters. There was thus less free α-Lac available for the MCSNP and consequently the amount of grafted α-Lac on the MCSNP surface decreased. Although we only demonstrate the significance of $X1$, $X3$, $X1X2X3$ and $X1X3$, all the coefficients were kept for the quadratic model elaboration in order to maximize prediction capability.

According to the previous results, the predictive model was given by the following equation:

$$\hat{Y} = 26.66 + 1.74X1 + 0.32X2 + 5.34X3 + 0.89X1X2 + 1.29X1X3 + 0.74X2X3 - 1.66X1X2X3 - 0.88(X1)^2 - 1.58(X2)^2 + 0.42(X3)^2$$

The predictive model allows predicting optimal conditions (Table 6).

According to the quadratic model, the maximum response value should be 34.5 α-Lac per MCSNP (data not shown). The experimental value for the response measured in optimum conditions was 34.1 α-Lac per MCSNP with a high reproducibility (RSD = 2%, 3 repetitions interday). The comparison of the two values showed that the predicted and experimental response values were non-statistically different ($t_{obs3} = 0.4 < t_{ref} = 3.2$) which underlined the good adequacy between quadratic model and experiments.

Fig. 4 represents the contour plot of the isoreponses around the optimal as a function of $X1$ and $X2$, $X3$ being set at +1. The arrow indicates optimal coordinates for $X1$ and $X2$. This isoresponse rep-

Table 5
Evaluation and significance of the quadratic model coefficients.

Factors	Coefficients	RSD	t_{obs}	P value
Constant	26.6643	0.6730	39.6200	<0.0001
$X3$	5.3400	0.5410	9.8700	<0.0001
$X1$	1.7400	0.5410	3.2200	0.0147
$X1X2X3$	−1.6625	0.6048	−2.7500	0.0286
$X1X3$	1.2875	0.6048	2.1300	0.0708
$X2X2$	−1.5786	1.0393	−1.5200	0.1726
$X1X2$	0.8875	0.6048	1.4700	0.1857
$X2X3$	0.7375	0.6048	1.2200	0.2622
$X1X1$	−0.8786	1.0393	−0.8500	0.4258
$X2$	0.3200	0.5410	0.5900	0.5728
$X3X3$	0.4214	1.0393	0.4100	0.6972

Table 6
Optimal conditions of α-Lactalbumin grafting according to the predictive model.

Factors	Optimal conditions	
	X	Real values
[EDC]/[α-Lac]	+1	25
[NHS]/[EDC]	+0.08	1.55
[α-Lac]	+1	24.85 nmol mL^{-1}

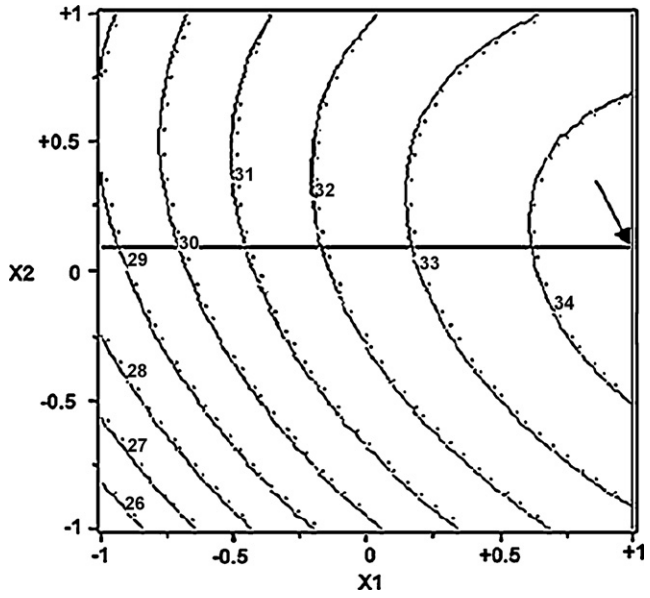

Fig. 4. Contour plot with isoresponses according to *X*1 and *X*2 in the near optimal domain with *X*3 = +1.

resentation with *X*1 and *X*2 was chosen as an example to show that the response variation when *X*1 and *X*2 vary is rather limited, which reflects grafting robustness [18] around optimal conditions. Slight increase of each factor ranging in the non-denaturing domain shows that the response does not increase any more, which could be explained by MCSNP surface saturation. Concerning grafting time dependence, the optimal grafting can be obtained after only 4 h of incubation (data not shown).

4.3. α-Lac biorecognition

After the optimization of α-Lac immobilization on MCSNP, the immunological recognition as well as colloidal stability were evaluated. First it was crucial to verify that α-Lac conformation was still recognized using specific Ab. Then we needed to find the best conditions to ensure a good grafted α-Lac/Ab interaction in solution which implies to keep MCSNPα-Lac colloidal behaviour. So we have tested different buffer composition and compared their influence on immune recognition and MCSNPα-Lac colloidal stability. Previous studies have shown that ionic strength ranging from 10 to 150 mM maintained MCSNP colloidal stability (data not shown). The chosen ionic strength was 50 mM to get a sufficient buffering capacity. Two parameters were then evaluated: buffer pH that was selected from 4 to 7.5 in order to surround α-Lac pI value (4.2–4.5), and buffer nature. Thus, after performing α-Lac optimum grafting, MCSNPα-Lac were solubilized in the buffers gathered in Table 7 in view of performing the immunoreactions with Ab. Fig. 5 shows the signal obtained using 10 ng mL^{-1} (final concentration) of specific or non-specific labelled Ab.

Table 7
Buffers used for immunoreactions and magnetic core–shell nanoparticles stability studies.

Buffers	Ionic strength (mM)	pH
Acetate	50	4
Formate	50	4
MES	50	6
PBS	50	7
HEPES	50	7.5
MOPS	50	7.5

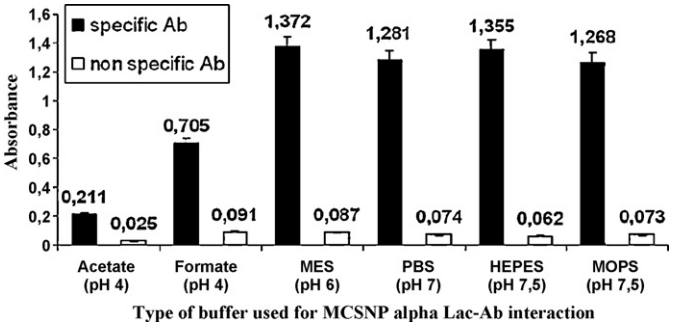

Fig. 5. Influence of pH and buffer nature on MCSNPα-Lac-Ab reaction. The experiments were performed by adding MCSNPα-Lac with specific or non-specific Ab for 2 h at 20 °C. After rinsing step, HRP conjugated Ab were detected by catalytic reaction using OPD substrate. Experiments were repeated 3 times (interday). See details in the text.

The specific Ab signal was 17-fold higher than non-specific Ab signal using buffers with pH from 6 to 7.5. These results demonstrated that grafted α-Lac was not denatured after covalent immobilization and that non-specific interaction was negligible. When performing the same experiments with non-grafted MCSNP with α-Lac, a signal of the same order of magnitude was observed for both specific and non-specific Ab. This indicates that Ab could interact with bare MCSNP surface probably due to electrostatic interaction with amino groups. These results demonstrated the necessity to saturate MCSNP with α-Lac in order to overlap amino moieties and thus to avoid non-specific interaction.

As expected, buffers of pH ranging from 6 to 7.5 lead to approximately the same specific signal whereas in acidic buffer, the signal dramatically decreased especially for acetate buffer which is a chaotropic buffer disfavoring immune interaction. In parallel, these buffers do not have significant influence concerning non-specific signal, except for acetate which prevents more efficiently non-specific interaction.

The grafted MCSNP colloidal stability was then studied in the course of time using the same buffers. Colloidal stability was followed by visual observation of aggregate formation. Acidic buffers at pH 4 maintained MCSNP colloidal behaviour at least for many weeks whereas buffer above pH 6 lead to MCSNP aggregation after 2 days of storage at 4 °C. Indeed MCSNPα-Lac charge depends on APTS amino groups and α-Lac charge. The pI of free α-Lac remains around 4.5, it can thus be assumed that at pH 4, this protein and amino groups are globally positively charged which leads to electrostatic repulsions providing MCSNP colloidal stability. On the contrary, at pH 7.5, the amino moieties were still protonated whereas α-Lac has some negative charges which could induce electrostatic interaction between MCSNPα-Lac favoring their aggregation. The results obtained with MES buffer at pH 6 are more surprising. Despite a pH value higher than free α-Lac pI, a satisfying MCSNPα-Lac stability was obtained at least during 7 days. A possible explanation could be that α-Lac structure and global charge were modified after immobilization, indeed α-Lac carboxylic group are involved in the covalent binding, in this way α-Lac becoming more basic, thus slightly modifying its pI. Finally, MES buffer appeared as a good compromise between Ab capture efficiency and MCSNPα-Lac stability.

In order to investigate further the influence of MCSNPα-Lac storage, immune response of grafted MCSNP after 7 days of storage was studied in MES and formate buffer which combine satisfying immunological capture and MCSNPα-Lac stability.

As illustrated in Fig. 6, the immobilized α-Lac stored in MES was still recognized by specific Ab even 7 days after grafting with a little decrease (14%) of the signal. The non-specific signal given by non-specific Ab was maintained at low level but doubled. These results underlined that immobilized α-Lac was slightly denatured after 1

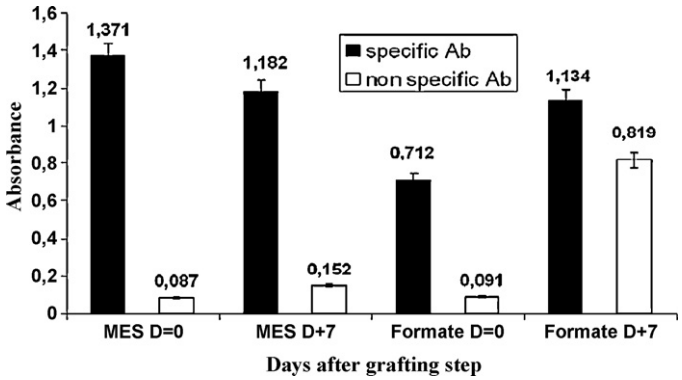

Fig. 6. α-Lac recognition at one time after immobilization step. The experiments were performed by adding MCSNPα-Lac with specific or non-specific Ab for 2 h at 20 °C the day of α-Lac immobilization ($D = 0$) or 7 days later α-Lac immobilization ($D + 7$). HRP conjugated Ab were detected by catalytic reaction using OPD substrate. Experiments were repeated 3 times (interday). See details in the text.

week of storage in 50 mM MES buffer at pH 6. A BCA test which allows α-Lac quantitation was performed on free α-Lac solubilized in 50 mM MES buffer after a space of 7 days and revealed a loss of 20% of signal which could confirm a modification in α-Lac structure. In formate we observed an increase of both signals obtained with specific and non-specific Ab. The increase of non-specific signal using non-specific Ab could be explained by the denaturation of grafted α-Lac which give access to MCSNP surface and thus favor non-specific interactions with Ab. As previously mentioned, a BCA test has also been performed on free α-Lac stored for 7 days in formate buffer. A decrease of 40% of the signal has been observed. It confirms the denaturation due to the buffer nature and underlined that this phenomenon is more important in formate than in MES. Concerning specific Ab reaction in formate, a part of the signal is certainly due to reaction between specific Ab and remaining native α-Lac, the other part of the signal is due to non-specific interaction with MCSNP surface.

It can thus be concluded that to achieve the best specific and sensitive immunological capture as well as keeping MCSNP colloidal stability, immunological reaction should be performed in 50 mM MES buffer at pH 6.

5. Conclusion

The use of experimental design allowed the optimization of α-Lac immobilization on MCSNP surface. This work was sequential. A 1st factorial design permitted to obtain a brief outline of factors influence on response variations and to determine an experimental domain that should contain the optimum. The subsequent central composite design was able to establish a predictive model in order to find out the optimal grafting coordinates which were experimentally verified as well as robustness. α-Lac biorecognition using anti-α-Lac antibody proved that the grafted protein was not denatured due to covalent immobilization and that α-Lac recognition was specific. The chemometric approaches allowed MCSNP surface saturation with α-Lac in non-denaturing conditions in order to efficiently capture specific anti-α-Lac antibody while avoiding non-specific antibody adsorption. Future works will consist in using MCSNPα-Lac in a microsystem scale in order to perform immunoassay dedicated to α-Lac allergy diagnosis in homogeneous liquid phase.

Acknowledgements

The authors thank the Agence national pour la recherche (ANR) for financial support. They also thank Bernard Malezieux for writing assistance.

References

[1] M. Slovakova, N. Minc, Z. Bilkova, C. Smadja, W. Faigle, C. Fütterer, M. Taverna, J.-L. Viovy, Lab. Chip 5 (2005) 935.
[2] G. Bayramoğlu, S. Kiralp, M. Yilmaz, L. Toppare, M.Y. Arıca, Biochem. Eng. J. 38 (2008) 180.
[3] M.E. Soderquist, A.G. Walton, J. Colloid Interface Sci. 75 (1980) 386.
[4] J. Li, X. He, Z. Wu, K. Wang, G. Shen, R. Yu, Anal. Chim. Acta 481 (2003) 191.
[5] C. Peskoller, R. Niessner, M. Seidel, J. Chromatogr. A 1216 (2009) 3794.
[6] A. Weissenbfck, M. Wirth, F. Gabor, J. Control. Release 99 (2004) 383.
[7] I.H. Cho, E.H. Paek, H. Lee, J.Y. Kang, T.S. Kim, S.H. Paek, Anal. Biochem. 365 (2007) 14.
[8] K. Sato, M. Yamanaka, T. Hagino, M. Tokeshi, H. Kimura, T. Kitamori, Lab. Chip 4 (2004) 570.
[9] K.S. Kim, J.K. Park, Lab. Chip 5 (2005) 657.
[10] J.W. Choi, K.W. Oh, J.H. Thomas, W.R. Heineman, H.B. Halsall, J.H. Nevin, A.J. Helmicki, H.T. Henderson, C.H. Ahn, Lab. Chip 2 (2002) 27.
[11] S.Y. Yang, K.Y. Lien, K.J. Huang, H.Y. Lei, G.B. Lee, Biosens. Bioelectron. 24 (2008) 855.
[12] J. Do, C.H. Ahn, Lab. Chip 8 (2008) 542.
[13] B.H. Hui, Z.J. Tsao, J.J. Wang, F.Y. Yu, Anal. Chem. 80 (2008) 7029.
[14] Y.J. Liu, D.J. Yao, H.Y. Chang, C.M. Liu, C. Chen, Biosens. Bioelectron. 24 (2008) 558.
[15] V. Maurice, T. Georgelin, J.M. Siaugue, V. Cabuil, J. Magn. Magn. Mater. 321 (2009) 1408.
[16] F. d'Orlyé, A. Varenne, T. Georgelin, J.M. Siaugue, B. Teste, S. Descroix, P. Gareil, Electrophoresis 30 (2009) 2572.
[17] J.V. Staros, R.W. Wright, D.M. Swingle, Anal. Biochem. 156 (1986) 220.
[18] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers Verbeke, Handbook of Chemometrics and Qualimetrics Part A, Elsevier, Amsterdam, 1997, pp. 643–658.
[19] R. Massart, J.C. Bacri, R. Perzynski, D. Salin, J. Magn. Magn. Mater. 62 (1986) 36.
[20] N. Fauconnier, A. Bee, J. Roger, J.N. Pons, Prog. Colloid Polym. Sci. 100 (1996) 212.
[21] Y. Lu, Y. Yin, B.T. Mayers, Y. Xia, Nano Lett. 2 (2002) 183.
[22] J. Vial, A. Jardy, Analusis 26 (1998) 15.
[23] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers Verbeke, Handbook of Chemometrics and Qualimetrics Part A, Elsevier, Amsterdam, 1997, pp. 263–303.
[24] J.J. Daudin, S. Robin, C. Vuillet, Statistique inférentielle, Presses Universitaires de Rennes, Société Française de Statistique, 2001, pp. 85–98.
[25] J. Sall, L. Creighton, A. Lehman, JMP Start Statistics, third ed., Thomson Books/Cole, Belmont, CA, USA, 2005, p. 397.