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Silica–dextran sorbent composites and their cleaning in place

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ABSTRACT

Large-scale liquid chromatography requires sorbents that are mechanically stable in order to ensure repeated cycles without shrinkage. The industrial separation of biological materials moreover implies working in apyrogenic and sterile conditions. Dextran-coated silica used as a chromatographic sorbent was treated with different sanitizing solutions after bacterial contamination. It was demonstrated that most of the strains used were inactivated by using alkaline solutions or ethanolic acetic acid. However, the sporulated form of *Bacillus subtilis*, which seemed more resistant than other strains, was inactivated only when alkaline solutions contained more than 40% ethanol. All these treatments were also very effective for pyrogen removal and did not indicate significant degradation of the silica moiety or the chromatographic performance of the sorbent.

INTRODUCTION

Among the different hydrophilic coated silicas that have been described, silica–dextran composites undoubtedly represent one of the major recent accomplishments in the design of solid sorbents for the liquid chromatography of proteins. Their superiority is the result of the association of a mineral skeleton with a biohydrocolloid, the first conferring remarkable mechanical resistance and the second avoiding the non-specific adsorption of proteins and bringing in the approriate chemical group. By this principle, ion-exchange sorbents [1,2], affinity sorbents [3,4] and hydrophobic sorbents have been synthesized and used in the separation of protein mixtures.

As one of the weaknesses of silica-based sorbents is their sensitivity at alkaline pH, a dextran coating seemed to be an appropriate solution to prevent too rapid solubilization of silica. In this respect, opinions on the stability of silica towards alkaline conditions did not seem very consistent in the literature and probably differed as a consequence of the variable nature of silica and of the coating (grafted silica). Some authors indicate that at pH > 7 the life of the packing material could be seriously reduced [5], whereas others [6,7] claimed that reversed-phase silica could remain stable at pH up to 9–10. Wehrli *et al.* [8] demonstrated that the rate of dissolution of silica by strongly alkaline solutions was very dependent on the content of organic modifiers. More recently, Law and Chan [9] concluded that, contrary to popular belief, silica-based packing materials can show excellent stability with certain alkaline eluents.

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The resistance of a sorbent against alkaline attack is in any case an important concern when the sorbents must be repeatedly used in clean conditions for preparative biological applications. The dextran moiety, widely used for more than 30 years, is well known for its good stability in sanitizing conditions (salt, temperature, strong alkaline solutions, etc.). In this paper, we first describe the fundamental properties of silica–dextran composites and their derivatives for use in liquid chromatography. A study focused on cleaning in place and sanitization is also reported, with particular reference to sterilization possibilities and their influence on the physico-chemical properties of the alkali-treated sorbent.

EXPERIMENTAL

Silica was obtained from IBF-Biotechnics (Villeneuve la Garenne, France) and dextran and dextran ionic derivatives from Sigma, (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade from Sigma or from Aldrich (Beerse, Belgium). Silica–dextran composite materials were synthesized according to previously described techniques [1,3,4].

Coating of porous silica with dextran

Dry silica beads with a pore volume of 1 cm^3/g and a mean pore size of 1200 Å were coated with cationic or anionic dextran. The dextran used had a molecular weight between 70 and 500 kilodalton and possessed a certain amount of ionic groups (about 0.5–1% for the synthesis of non-ionic derivatives and about 10% for the preparation of ion exchangers). Briefly, the coating was performed as follows: 25 g of dextran derivative were dissolved in 150 ml of 0.2 *M* carbonate buffer (pH 10.5), then 5 ml of butanediol diglycidyl ether were added with stirring. Finally 100 g of dry silica beads were added (specific surface area 25 m^2/g; pore diameter 1250 Å). The suspension was then filtered under vacuum to remove the excess of the dextran solution. The gel was placed in a ventilated oven and kept at 80°C for 24 h. The dry product obtained was repeatedly washed with water and with acidic and alkaline solutions, and finally stored in the presence of 1 *M* sodium chloride.

Sanitization treatments

Dextran-coated silica was first contaminated with a large amount of a defined microorganism (*Escherichia coli, Staphylococcus aureus, Candida albicans* and the sporulated form of *Bacillus subtilis*) and then decontaminated by washing with appropriate solutions.

The solutions used were sodium hydroxide at different concentrations, 60% ethanol–0.5 *M* acetic acid with different incubation times and mixtures of 0.2 *M* sodium hydroxide with ethanol in different proportions.

After contamination, the sorbents were treated with three volumes of the appropriate sanitizing solution (up to 24 h at 25°C). The remaining germs were then detected on the neutralized sorbent slurry using a standard culture. Tests were performed in diluted tryptase–soya sterile broth in triplicate per dilution. Each tube was incubated at 33°C for 14 days with daily observations; the number of germs was determined classically according to the original Sperman–Karber method [10,11] as recommended by the World Health Organization [12].

Stability studies of silica–dextran sorbents

Two kind of studies were performed: (i) physico-chemical modifications of the silica-dextran matrix with ion exchangers and (ii) modification of the performance of a packed column after numerous strong alkali treatments.

In the former instance, ion-exchange dextran-coated silica was treated in a stirred suspension with sodium hydroxide from 0.1 to 1 *M* for 1 to 24 h. Each sample was then tested in order to determine the amount of leached silica (in the supernatant) and to measure the modifications of the specific surface area (BET) and the pore diameter (mercury porosimetry). Leached silicon was determined by elemental analysis.

In the latter instance, an analytical column of 8 ml (10 cm × 1 cm I.D.) of dextran sulphate-coated silica was submitted to 250 washings with 2 *M* sodium hydroxide at room temperature. The volume of alkaline solution injected was 0.5 ml per cycle. These treatments were followed by equilibration with 0.05 *M* acetate buffer (pH 4.5) and by the chromatographic separation of a mixture of β-lactoglubulin and cytochrome *c*. Then proteins were eluted under a salt gradient up to 0.8 *M*. To determine the possible influence of these sodium hydroxide washings, the chromatographic resolution was calculated.

RESULTS AND DISCUSSION

Sorbent synthesis and properties

Coating porous silica beads with dextran or ionic dextran derivatives is necessary for eliminating the well know non-specific protein adsorptions or to introduce ion-exchange properties. Indeed, on native silica, most proteins are tightly adsorbed and frequently denatured. As the synthesis of silica ion exchangers and their properties have been extensively described elsewhere [1,2], our study was focused on non-ionic silica–dextran composites.

It should be noted that a complete coating with a polysaccharide prevented direct contact between the protein and the silanol groups; this is clearly evidenced (Table I) by the progressive decrease in the adsorption of a cationic protein such as cytochrome *c*. An increase in the dextran content lowered to zero the interaction between cytochrome *c* and the silica gel. The dextran layer also contributes to making the silica more resistant to strongly alkaline media and further makes easy the acti-

TABLE I

EFFECT OF DEXTRAN COATING ON THE SORPTION CAPACITY OF CYTOCHROME *c* AND ON THE V_e/V_t RATIO OR BSA

Amount of dextran (g per 100 g silica)	Cytochrome *c* sorption capacity (mg/ml)	V_e/V_t[a] (BSA)
5	3.7	0.77
10	2.5	0.71
15	1.1	N.D.
20	0.2	0.60

[a] Experiments performed on-column (V_t = total volume of the column; V_e = elution volume).

vation of polysaccharide-based supports involving the classical reagents used in affinity chromatography. Altough a high concentration of dextran is beneficial to the elimination of non-specific adsorptions, it also diminishes the porosity of the support. The value of V_e/V_t (ratio of the elution volume for bovine serum albumin (BSA) to the total column volume) descreased when the amount of dextran increased. This decrease is linked with the progressive reduction of the pore size inside the silica network. The degree of cross-linking (different amounts of cross-linking agent) did not significantly modify the behaviour of the silica–dextran composite towards cytochrome *c* and BSA (data not shown).

Non-ionic derivatives can be easily used in affinity chromatography after activation and immobilization of biologically active substances such as concanavalin A and protein A [3,4,13].

Sterilization results

It was found that the inactivation of germs by sodium hydroxide washing was dependent on the nature of the strains used (see Table II). This is the reason why we used the strain that covers fairly well the range of common microorganisms such as bacteria, fungi and other sporulated standard forms (*e.g., Bacillus subtilis*). The latter is recommended by pharmacopoeias for validating cleaning operations. In this study, *Escherichia coli* seemed the most sensitive to alkaline media; it was in fact totally inactivated by sodium hydroxide at concentrations as low as 0.05 *M*. *Candida albicans* and *Staphylococcus aureus* were also sensitive to sodium hydroxide treatment but their total inactivation was observed when the sodium hydroxide concentration was 0.1 *M* or higher. However, sodium hydroxide at any concentration (and at temperatures of 20–25°C for 3 h) was not found to be very effective in the inactivation of the sporulated form of *Bacillus subtilis*, even when the sodium hydroxide treatment was extended up to 24 h. In the best instances, the decrease in the amount of *Bacillus subtilis* was about five $\log_{10}$, which means that, from an initial concentration of about 10^7 germs/ml, about 100 germs/ml were still present at the end of the treatment (0.2 and 1 *M* sodium hydroxide).

TABLE II

GERM IN ACTIVATION USING DIFFERENT CLEANING SOLUTIONS (3 h, ROOM TEMPERATURE)

Washing solution	*E. coli* (Germs/ml gel)	*S. aureus* (germs/ml gel)	*C. albicans* (germs/ml gel)	*B. subtilis* (germs/ml gel)
Before washing	10^7	10^7	10^7	10^7
0.2 *M* NaOH	0	0	0	200
1 *M* NaOH	0	0	0	70
60% Ethanol–0.5 *M* acetic acid	0	0	0	800
20% Ethanol–0.2 *M* sodium hydroxide	0	0	0	20
40% Ethanol–0.2 *M* sodium hydroxide	0	0	0	3
60% Ethanol–0.2 *M* sodium hydroxide	0	0	0	0

The treatment of contaminated supports with 60% ethanol–0.5 *M* acetic acid indicated an extreme sensitivity of all the strains studied, except *Bacillus subtilis* (see Table II). Whereas with ethanol–acetic acid treatment for less than 1 h *E. coli, C. albicans* and *S. aureus* germs were quantitatively inactivated, the number of *B. subtilis* decreased greatly during the first few hours of treatment (four $\log_{10}$) and then very slowly so that after 24 h of treatment a significant level of contamination (180 germs/ml) still persisted (data not shown).

These results are in accordance with those presented by Whitehouse and Clegg [14], who reported a strong resistance of *B. subtilis* towards treatment with sodium hydroxide. It was demonstrated that at 22°C, 1 *M* sodium hydroxide decreased the initial amount of *B. subtilis* by about four $\log_{10}$ in 12 h whereas under the same conditions it took 25, 49 and about 70 h to obtain similar results with 0.6, 0.4 and 0.2 *M* sodium hydroxide, respectively.

It was found, however, that alternate treatment of a contaminated sorbent for 1.5 h each with 0.2 *M* sodium hydroxide and ethanol–acetic acid totally inactivated *B. subtilis*. These results led us to investigate treatment with a solution composed of 0.2 *M* sodium hydroxide and ethanol at concentrations between 20 and 60% for 3 h. When the ethanol concentration was above 40%, the inactivation of sporulated *B. subtilis* was total. However, with an ethanol concentration of 20%, although sterilization of contaminated sorbents was very good for *E. coli, C. albicans* and *S. aureus, B. subtilis* was not totally destroyed (see Table II). From these results, it can be concluded that, if total elimination of germs in a chromatographic support is to be achieved, it is necessary to apply either alternate treatment with 0.2 *M* sodium hydroxide and ethanol–acetic acid or an alkaline treatment at low concentration in the presence of ethanol.

When simple elimination of pyrogens is required, all the above-mentioned methods could be used. In this situation, three general problems could occur on the practical level: (1) with such treatments in the presence of ethanol, soft or semi-rigid gels may shrink to various extents, thus making operation on-column very difficult; (ii) the solid matrix could be damaged during the treatments; (iii) the stability of the biologically active ligand attached to the sorbents may be affected. It is clear that with rigid sorbents such as dextran-coated silica, all treatments with high concentrations of ethanol could be applied without any shrinkage or channelling.

Sodium hydroxide resistance of silica–dextran composites

The resistance of dextran-coated silica was studied when using alkaline solutions. It is known that silica is particularly stable in acidic media. Further, in the presence of 0.5 *M* acetic acid, the cross-linked dextran moiety, whether chemically derivatized or not, is also fairly stable.

It is currently considered that silica-based material will be progressively destroyed by the use of strongly alkaline aqueous solutions. However, only a few papers have reported experimental results on the behaviour of the silica packing when washed at alkaline pH [5–9].

In most instances, the packing material was a reversed-phase silica with C_8 and C_{18} aliphatic chains chemically bonded on the silica material and the degradation was determined by the loss of organic coating. The use of organic modifiers such as acetonitrile or methanol in the alkaline solution showed that the dissolution was reduced for non-bonded silica [8].

In this study, silica was coated with a cross-linked cationic macromolecule, the association between the two entities being dependent on the strong ionic multi-point exchange. To determine the degradation of this particular sorbent, we used the elemental analysis of the released silicon because the organic layer was neither hydrolysed nor solubilized or dissociated from the matrix.

Experimental data showed that an alkaline treatment released small amounts of silicon from the sorbent. This limited silica solubilization was dependent on the sodium hydroxide concentration and on the treatment time (see Table III). It was found that 0.2 *M* sodium hydroxide, classically used in the sanitization of these sorbents (see above), induced the solubilization of about 0.3% of silica after a 2-h treatment. When the treatment was continued for 10 h, the silica solubilization was *ca.* 2%.

These chemical phenomena did not indicate any modification of the chromatographic behaviour, *e.g.*, sorption capacity, titration curve and separation efficiency of ion exchangers based on silica–dextran composite materials, which are all dependent on the properties of the coating material.

The specific surface area varied only very slightly when the sodium hydroxide concentration was below 0.1 *M*; at 0.2 *M* sodium hydroxide, it increased by *ca.* 5–10% and then remained constant. This was checked with treatments of 1 and 24 h (Table IV).

The pore diameter remained almost constant when the alkaline treatment was limited (*e.g.*, 1 h). For 24-h treatment, the pore diameter was modified when the sodium hydroxide concentration was increased from 0.1 to 0.2 *M*; higher concentrations did not show any clear modification of this parameter, except at 1 *M* for 24 h.

In summary, the specific surface area seemed not to be significantly affected by sodium hydroxide treatments (Table IV). Beyond 16-h pefusion, silica-dextran composites can be progressively damaged particularly when the sodium hydroxide concentration is very high. It should be kept in mind, however, that sodium hydroxide concentrations above 0.2 *M* are not necessarily suitable for the total sanitization of the sorbents (see above). On the other hand, for cleaning purposes only, when the sorbent becomes contaminated owing to the injection of very crude samples, sodium hydroxide at concentrations up to 2 *M* could be the sole means of regenerating correctly.

Under these conditions, it was demonstrated that very numerous repeated sodium hydroxide treatments did not significantly decrease the chromatographic effi-

TABLE III

EFFECT OF SODIUM HYDROXIDE TREATMENT ON THE SOLUBILIZATION OF SILICON FROM DEXTRAN-COATED SILICA

Sodium hydroxide concentration (*M*)	Silicon solubilized (%)		
	after 1 h	after 5 h	after 15 h
0.1	10.18	–	–
0.2	0.40	0.98	2.78
0.5	0.41	–	–
1.0	0.52	–	–

TABLE IV

VARIATION OF THE SPECIFIC SURFACE AREA AND OF THE MEAN PORE DIAMETER AS A FUNCTION OF SODIUM HYDROXIDE TREATMENT OF DEXTRAN-COATED SILICA

Sodium hydroxide concentration (*M*)	Specific surface area (m^2/g)		Mean pore diameter (Å)	
	1 h	24 h	1 h	24 h
Before treatment	24	24	1000	1000
0.1	24	25	1000	1220
0.2	25	26.5	980	1250
0.4	25.5	27	980	1250
0.8	25.5	27	1000	1300
1.0	25.3	27	1050	1600

ciency of the sorbent. Fig. 1 shows that the reslution decreased slightly after each cycle. After 250 cycles of washing with 2 *M* sodium hydroxide for 8–10 minutes each (this corresponds to a total time of treatment of 33–42 h at room temperature) the total decrease in resolution was only 16–18% and was approximately 0.07% per cycle (average). During this very drastic treatment the column back-pressure did not change significantly and the length of the column bed decreased only by less than 1%. Even though this last comment is not related to the inactivation protocols, it shows that when a sterilized chromatographic packing is not totally cleaned up (adsorbed material), additional washings with 1–2 *M* sodium hydroxide can be performed. Conversely, these treatments are not efficient enough for proper sterilization even after several hours.

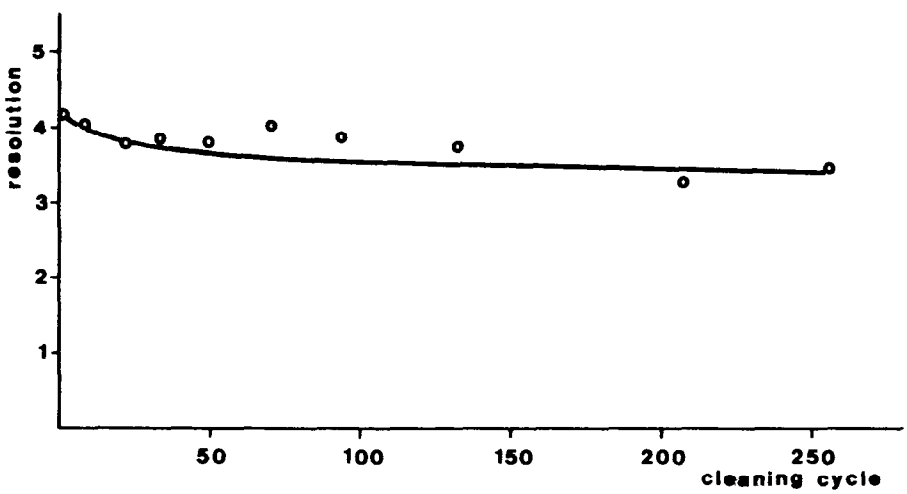

Fig. 1. Influence of 2 *M* sodium hydroxide cleaning in place on the resolution of a column of dextran sulphate–silica packing (S-Zephyr). Column, 10 cm × 1 cm I.D. (7.8 ml); particle size, 20 ± 5 μm; buffer, 0.05 *M* acetate (pH 4.5) (sodium chloride gradient); protein separated, β-lactoglobulin and cytochrome *c*; separation time, 25 min (including 8–10 min of cleaning with 2 *M* sodium hydroxide); working pressure, 15 bar.

CONCLUSIONS

Although the necessity to clean all types of sorbent (ion exchangers, gel filtration and affinity media) is undoubtedly admitted, the real efficiency and the consequences of the chemical treatments were not clearly known in the past. This study has demonstrated that sodium hydroxide treatment does not guarantee good sterilization. *Bacillus subtilis* was in fact not totally inactivated by alkaline treatment, whatever the period of contact or the sodium hydroxide concentration, and an additional ethanol–acetic acid treatment was necessary to eliminate any germs. This result was also achieved when the sterilization was effected with a mixture of sodium hydroxide and ethanol.

These treatments are particularly suitable for dextran-coated silica, which is incompressible in the presence of any amount of ethanol. It was in addition demonstrated that these sorbents were particularly stable when treated with alkaline solutions. Only a very small amount of silica was dissolved, permitting repeated concentrated (2 *M*) sodium hydroxide washings without significant modification of the chromatographic properties of the sorbents.

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