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Biofunctional polystyrene derivatives exhibit a high affinity for the epidermal growth factor-receptor (EGF-R): Part I

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Insoluble sulfamide of amino acid polystyrene derivatives interact with solubilized epidermal growth factor receptor (EGF-R). The choice of the different amino acids substituted on the polystyrene macromolecular chain is governed by the nature of the peptide involved in the binding of EGF on its receptor. This binding has a hydrophobic character with two affinity constants for EGF-R. Some of the amino acids involved in this fixation are known: Leu 44, Tyr 29, Leu 26, Ileu 23, Tyr 22, Val 19, Met 21. Therefore, statistic biofunctional polystyrene derivatives were synthesized with variable degrees of substitution by leucine and/or benzylamine or tyrosine. The random substitution may lead to complex sites that would interact with EGF-R like EGF. Indeed, among the studied copolymers, sulfamide of leucine (15% and 44% or tyrosine (35%) polystyrene derivatives exhibit a strong affinity for high and/or low affinity solubilized EGF-R and could be held as EGF-like polymers (affinity constants ranging from 2×10^9 to 2×10^{11} M^{-1})

1. Introduction

EGF is a potent polypeptide growth factor for a variety of animal cells. The mitogenic effect is exerted following its binding to a specific membrane receptor, EGF-R.

The EGF receptor is a MW 170000 and 1210 amino acids transmembrane glycoprotein that comprises various domains [1, 2]: an external EGF binding domain which is a cysteine rich sequence of 621 amino acids containing the sites for N-glycosylation, a transmembrane domain containing 23 amino acids and an internal domain of 542 amino acids that contains the tyrosine kinase site and the major autophosphorylation sites located in the COOH terminal segment.

Several groups have shown that most cell lines exhibit two types of EGF receptors *id est* two classes of affinity towards EGF: a minor population of high-affinity EGF receptors ($Ka = 4 \times 10^9\ M^{-1}$) and a major population of low-affinity EGF receptors ($Ka = 1 \times 10^8\ M^{-1}$) [3–5]. Some authors correlate a monomeric state of the receptor with the low affinity class and an oligomeric state with the high affinity class [3, 6]. This question is not fully resolved.

Furthermore, studies reveal that abnormal expression of the EGF-R appears to be involved in uncontrolled cell growth and oncogenesis [7].

The numerous results obtained in experimental and clinical oncology direct studies towards the assessment of EGF-R as a prognostic factor in breast cancers [8, 9]. The absence of standardization of tissue processing and the poor reliability of assay conditions in tumors make difficult any comparisons between conclusions drawn from these studies. The role of each class of EGF-R in the oncogenesis process remains unknown. To answer this question, a purification of each EGF-R population is necessary.

First of all, we developed an immunoenzymetric assay for the low affinity EGF-R population. Then, the purpose of our work was to synthesize functional polystyrene derivatives whose macromolecular chains are substituted with variable percentages by benzylamine, leucine, tyrosine, these being some of the amino acids involved in EGF binding on its receptor. Random substitution on a polymer with favourable composition may lead to complex sites that would interact with EGF-R like EGF.

The adsorption of a solubilized EGF-R (ssEGF-R), extracted from human epidermoid cell line, A431, on theses resins was performed in static conditions (batches) and measured by using an immunoenzymetric assay; this phenomenon was analysed according to the Langmuir law.

2. Materials and methods

2.1. Synthesis of statistic biofunctional copolymers

Spherical crosslinked polystyrene beads (Biobeads SX2, Biorad Laboratories) were successively washed in 1 M NaOH, 1 M NaCl and distilled water, and dried under vacuum at 50 °C. Chlorosulfonation was carried out according to a method derived from Godivan *et al.* [10].

10 g of beads were swollen overnight in 300 ml dichloromethane at room temperature. Subsequently,

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an excess of 70 ml chlorosulfonic acid, and 30 ml dichloromethane were added and the suspension was stirred at room temperature for 1 h. The resin was then filtered in a sintered glass funnel and successively rinsed with dichloromethane, acetone and dichloromethane again. The resulting resin, $PSSO_2Cl$, was then used to obtain the different statistical functional polymers.

Sodium sulfonate polystyrene

Sulfonated polymers were hydrolysed successively with 0.1 M and 2 M NaOH and neutralized by washing steps in 0.01 M NaOH. The resulting polymer, $PSSO_3Na$, was then dried under vacuum at 50 °C.

Sulfamide of amino acid polystyrene

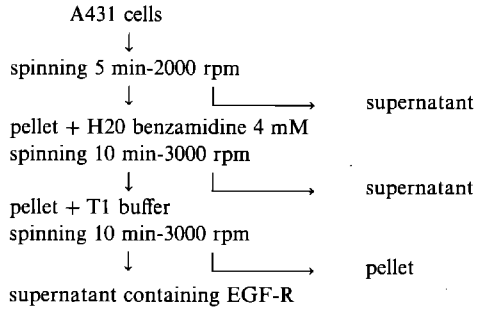
X mol of L-methyl ester Leucine or L-methyl ester tyrosine and *x* mol of triethylamin were mixed to 250 ml dichloromethane. $PSSO_2Cl$ resin was then introduced. After a few minutes, *x* mol of triethylamin were added. The suspension was stirred at room temperature for 48 h. The resulting polymer was successively washed with ethanol and 0.01 M NaOH and 2 M NaOH. The sulfamide of amino acid polystyrene derivative was then dried under vacuum at 50 °C. A standard curve, substitution rate in amino against the amount of introduced amino acid was established.

2.2. A431 cell culture

The A431 cell line was purchased from ATCC (CRL 1555). They were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal calf serum glutamine and antibiotics, at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Extraction of EGF receptors

A431 cells were harvested from 162 cm^2 tissue culture flasks (Costar) by incubation for 10 min at 37 °C with 4 ml phosphate-buffered saline (PBS)-EDTA (pH = 7.4). The cells were centrifuged and resuspended in DMEM culture medium and the cell concentration was determined using a Coulter Counter ZM. The extraction of EGF receptor was performed as follows [11]:



A431 cells lysed in a hypotonic medium (H_2O-benzamidine 4 mM) on ice for 5 min. The resulting homogenate was centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant containing the cytosolic fraction was discarded. The pellet constituted of membrane fractions was solubilized on ice in a solubilizing buffer (T1 : 10 mM Hepes, 4 mM benzamidine, 3 mM EDTA, 1 mM dithiotreitol, 1% Triton X-100).

After centrifugation at 3000 rpm for 10 min, the supernatant containing membrane solubilizate was collected and its ssEGF-R concentration determined by IEMA. Then aliquots of this membrane solubilizate were rapidly frozen and stored at − 20 °C. The pellet containing insoluble detergent was discarded.

2.4. Adsorption of solubilized EGF-R on polymers.

Passivation of resins

200 mg of beads were introduced in 5 ml PBS-bovine serum albumin (BSA 0.4 g/l). The suspension was stirred at room temperature for 30 min; then three washes were performed as follows:

After a sedimentation by gravity, the supernatant was eliminated, then 5 ml PBS were added, and the suspension was stirred at room temperature. After the third washing, resins in suspension in 5 ml PBS were stored at 4 °C.

Incubation of polymers with solubilized EGF-R

At 4 °C, 250 µl of beads were deposited in a passivated polypropylene tube; after sedimentation of the resin, 150 µl of supernatant were discarded, then 100 µl of solubilized EGF receptors at different known concentrations were added. The tubes were stirred for 30 min at 4 °C. The supernatants were removed and assayed for EGF-R.

2.5. Immunoenzymetric assay (IEMA) of solubilized EGF-R

Briefly, a 96-well microtiter plate was coated with an anti mouse IgM (from goat) monoclonal antibody by a 2 h incubation at 37 °C with 100 µl of a 2.5 µg/ml solution in PBS, pH = 7.4. Unoccupied binding sites were blocked by incubation overnight at 4 °C with 200 µl of a buffer containing 0.3% BSA.

After three washes with an assay buffer (10 mM Hepes, 0.1% BSA, 0.1% Tween 20, 150 mM NaCl), 100 µl of an anti EGF-R monoclonal antibody (mAbl) were added to the wells and incubated for 1 h at 37 °C.

After three additional washes, the plates were incubated successively with

20 µl of incubation buffer

30 µl of the sample to be assayed for EGF-R

50 µl of an anti EGF-R monoclonal antibody: mAb2 or mAb3 for 2 h at 37 °C.

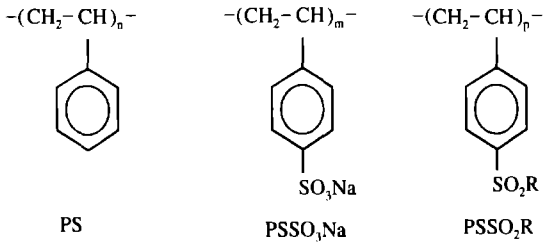
After six washes, the plates were incubated with a 3000-fold dilution of a peroxidase conjugated anti mouse IgG2b (from goat) monoclonal antibody for 1 h at 37 °C. Enzyme activity was determined using O-phenylenediamine as substrate and absorbance was measured at 490 nm in an microtiter plate reader (Biotek, EL 311). A standard curve giving absorbance at 490 nm against EGF-R concentration

was previously determined. For this, calibrated standards of EGF-R were used.

3. Results

3.1. Sulfamide of amino acids derivatives

The macromolecular chain of each synthesized copolymer is constituted of the three following monomers at various percentages:



R = Leucine or/and benzylamine, or tyrosine.

The percentage of monomer units was determined by elemental analysis for S, N and Na. The results are shown in Table I in the case of sulfamide of leucine derivatives.

3.2. Standards curves for EGF-R IEMA

The standard curves for EGF-R IEMA shown in Figs 1 and 2 are the mean of 12 duplicate assays with different calibrated standards. The concentration of ssEGF-R used for the establishment of standard curves varied from 0 to 2.5 nM and from 0 to 0.125 nM, respectively, for the low affinity class and high affinity class.

Within this concentration range, a linear correlation is obtained between the absorbance at 490 nm and the ssEGF-R concentration.

TABLE I Final composition of the synthesized polymers

S (meq/g)	N (meq/g)	Na (meq/g)	Final composition (%) of the copolymer		
			PS	$PSSO_3Na$	$PSSO_2Leu$
3.98	—	3.59	30	70	0
4.00	0.25	4.10	26	70	5
3.98	0.52	3.04	23	67	10
3.73	0.69	3.99	25	61	14
3.50	0.79	3.40	34	51	15
2.94	1.32	—	45	30	25
3.26	1.44	3.25	33	37	30
3.09	1.96	2.87	31	25	44
3.24	2.13	—	21	27	52
3.04	2.53	3.05	22	13	65

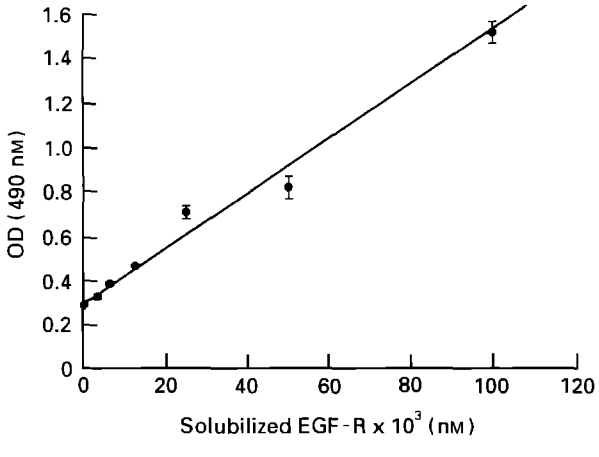


Figure 1 Standard curve for high affinity ssEGF-R IEMA

3.3. Adsorption of EGF-R on polymers

As mentioned above in materials and methods, the adsorption of solubilized EGF-R on polystyrene derivatives was quantified from a "by difference" assay; thus the concentration of ssEGF-R was determined before and after the incubation with the polymer.

Therefore, the experimental parameters obtained are the incubation concentration of ssEGF-R (Ci) and the free concentration (F) of ssEGF-R after incubation. By difference, we determined the concentration of ssEGF-R bound to the polymer (B).

The results are exploited according to the Langmuir law; as shown, for instance in Fig. 3, an adsorption isotherm of solubilized EGF-R on resins was performed for each polymer studied; thus, the concentration of bound ssEGF-R was plotted against the incubated EGF-R concentration. In this example, we notice that the concentration of bound ssEGF-R varies with the incubation concentration of ssEGF-R; a saturation plateau is reached towards Ci = 2 nM.

Scatchard (Fig. 4) and Langmuir (Fig. 5) analysis plots give dissociation constants and the maximal

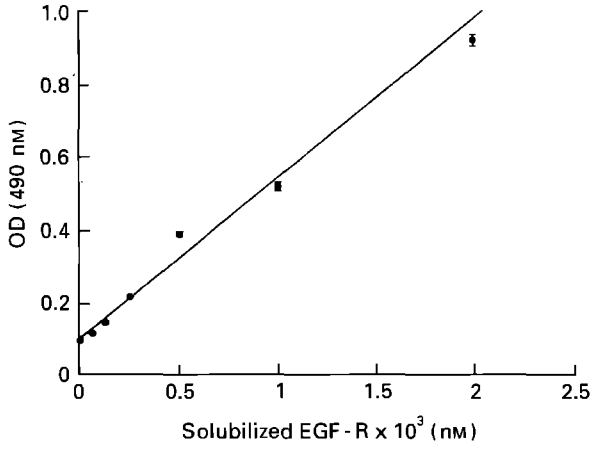


Figure 2 Standard curve for low affinity ssEGF-R IEMA

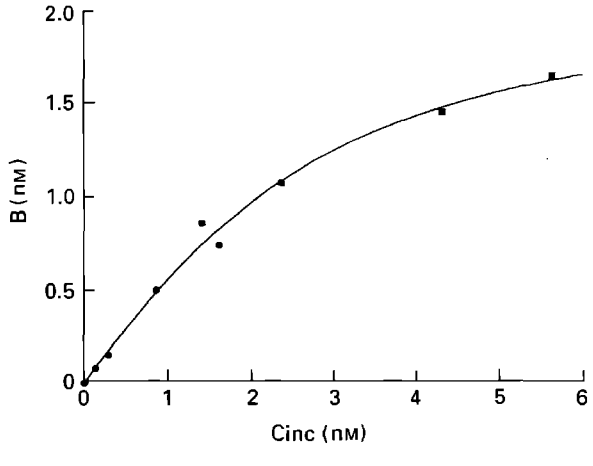


Figure 3 Adsorption isotherm of ssEGF-R on the polymer whose composition is $PS_{23}SO_3Na_{67}Leu_{10}$

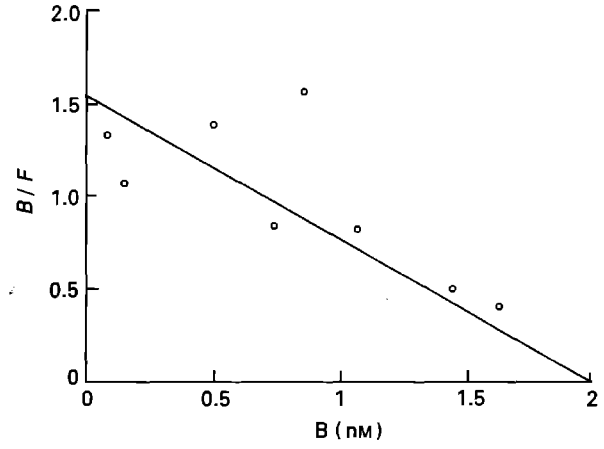


Figure 4 Scatchard representation B (Bound ssEGF-R concentration to the polymer) versus F (free ssEGF-R concentration)

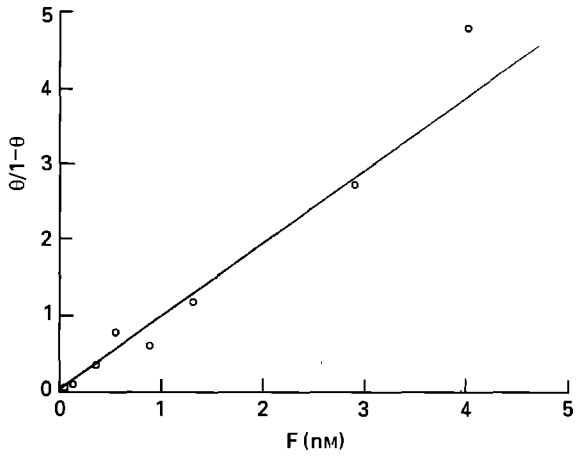
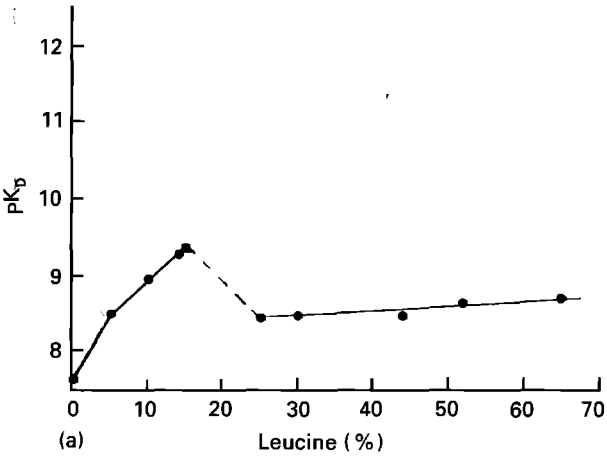


Figure 5 Langmuir representation; $\Theta = B/B_{max}$; B_{max} = maximal capacity of ssEGF-R adsorption on the polymer



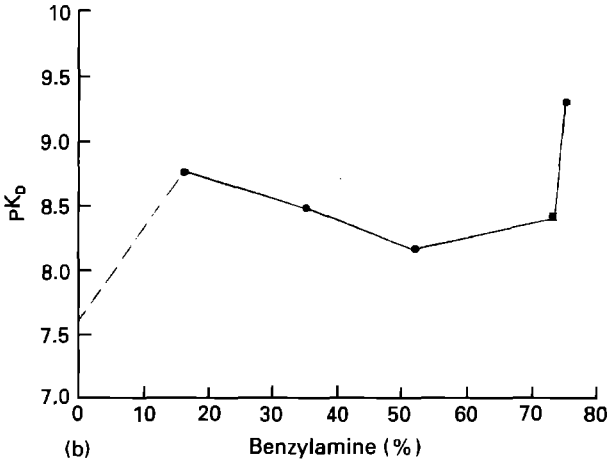
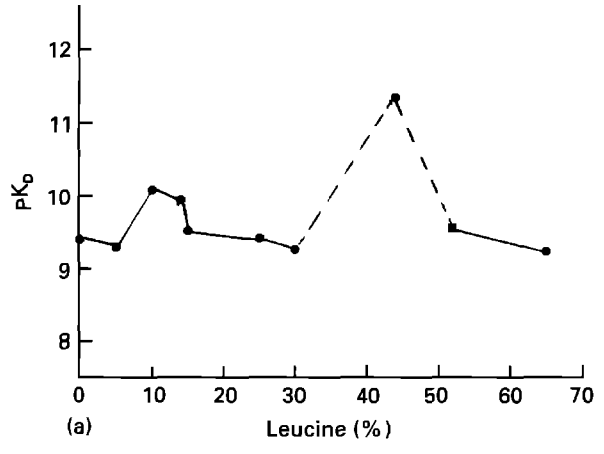


Figure 6 Influence of the subtitution rate in leucine or benzylamine on the affinity of statistic polymers towards low affinity ssEGF-R: (a) variation of affinity versus the substitution rate in leucine; (b) variation of affinity versus the substitution rate in benzylamine.



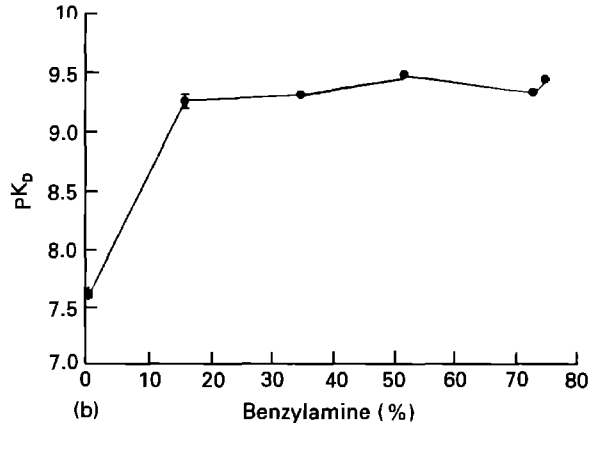


Figure 7 Influence of subtitution rate in leucine or benzylamine on the affinity of statistic polymers towards high affinity ssEGF-R: (a) variation of affinity versus the substitution rate in leucine; (b) variation of affinity versus the substitution rate in benzylamine.

capacity of EGF-R adsorption on polymers. For each polymer, successive iterations of the three graphic representations are necessary until the theorical points fit with experimental points.

To show the affinity variation of sulfamide of amino acids polystyrene derivatives towards ssEGF-R, we have chosen to plot the pKd of the polymer for ssEGF-R versus the substitution rate in amino acids of the polymers. As shown in Figs 6 and 7, the affinity of the polymers towards each class of EGF-R varies according to the composition of the resins.

Low affinity EGF-R

The pK_D of sodium sulfonate polystyrene ($PSSO_3Na$) is 7.5, thus has an affinity constant of $4 \times 10^7\ M^{-1}$. The pK_D increases with the introduction of leucine groups to reach a peak toward about 15% of leucine; this adsorption peak corresponds to a pK_D value of 9.5 and to an affinity constant 200-fold superior to the value obtained for $PSSO_3Na$ (Fig. 6a). From the substitution rate in leucine groups of 25%, the pK_D value is equal to 8.5 and remains constant.

In the case of sulfamide of benzylamine derivatives (Fig. 6b), the variation of affinity towards ssEGF-R is much less pronounced (pK_D ranging from 8.5 to 9.2 and affinity constants from 1.5×10^8 to 2.1×10^9 M^{-1}).

High affinity EGF-R

Two adsorption peaks are observed in the case of sulfamide of leucine polystyrene derivatives. The first peak appears at about 15% of leucine and the second one at about 45%; this latter peak corresponds to a pK_D value of 11.5 or an affinity constant of 2.2×10^{11} M^{-1}.

In contrast, we see no variation of affinity for sulfamide of benzylamine derivatives whatever the substitution rate.

4. Discussion

As shown above, the affinity of sulfamide of amino acids derivatives for EGF-R varies according to the substitution rate of the polymer.

The resins whose macromolecular chains are substituted with about 15% and 45% of leucine exhibit affinity constants equal to 2×10^9 M^{-1} and 2×10^{11} M^{-1}, respectively, for low affinity and high affinity EGF receptors. These affinity constants are superior to those obtained for EGF binding on its receptor at the membrane surface of A431 cells ($K_1 = 1 \times 10^8$ M^{-1} towards low affinity EGF-R; $K_2 = 4 \times 10^9$ M^{-1} towards high affinity EGF-R).

It must be noticed that the polymers used for the experiments were, first of all, passivated with BSA in order to block and to eliminate non-specific binding sites (affinity constant of BSA towards polystyrene derivatives: 10^5–10^6 M^{-1}). Therefore, the available sites are those which can develop an affinity interaction with ssEGF-R.

The maximal capacity of ssEGF-R adsorption on resins was also determined for each polymer (data not shown). No correlation between the maximal capacity of ssEGF-R adsorption and the substitution rate of the polymer was established.

We can assume that the specificity of the interaction between ssEGF-R and a given resin rests on to the conformation of the protein adsorbed on the polymer.

It may appear surprising that the presence of only one amino acid on the macromolecular chain leads to a so strong affinity for EGF-R. In fact, random substitution on the polystyrene seems to lead to different configurations of leucine sites and in particular to a configuration close to the one present on EGF; therefore, these polymers interact specifically with EGF-R.

In the case of sulfamide of benzylamine polystyrene derivatives, the variation of affinity towards ssEGF-R is much less pronounced; this means that the benzylamine group is not specifically involved in the binding with ssEGF-R.

As previously mentioned, the aromatic cores of Tyr-22 and Tyr-29 are implicated in the binding of EGF on its receptor, nevertheless, the presence of a hydroxyl group on the tyrosine changes the electronic distribution of the aromatic core and so, its properties. To check this assumption, adsorptions of ssEGF-R on sulfamide of tyrosine polystyrene derivatives have been carried out; the preliminary results are promising and confirm our hypothesis (data not shown).

The experiments that we have achieved do not allow us to determine the nature of interaction and the site involved in the adsorption phenomena. For this purpose, a series of radio ligand assays have been performed using ^{125}I-EGF to prevent or not its binding on the receptor which was pre-adsorbed at the polymer surface.

As with other anterior studies and works [12–14], this model illustrates the Jozefonvicz and Jozefowicz principle [15]. Indeed, the random substitution on a resin may lead to polymers which exhibit a favourable composition to complex sites that would interact with EGF-R like EGF. The probability of the occurence of biospecifics sites depends on the substitution rate of the macromolecular chains, *id est* the final composition of the copolymer.

Nevertheless, to control the EGF-like feature of these resins, a series of biological tests is to be performed, such as tyrosine kinase activity assay and their use as a micro carriers for cell culture, in order to modulate the expression of receptors.

If these tests prove to be fruitful, numerous applications of these polymers could be considered. On the one hand, they could be used in affinity chromatography for the purification of each class of EGF-R and on the other hand, within the framework of new therapeutic approaches such as cellular targeting.

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