

## EUROPEAN QUALIFYING EXAMINATION 2015

# Paper A(Ch)

## Chemistry

This paper comprises:

- |                             |                     |
|-----------------------------|---------------------|
| * Letter from the applicant | 2015/A(Ch)/EN/1-7   |
| * Document D1               | 2015/A(Ch)/EN/8-9   |
| * Document D2               | 2015/A(Ch)/EN/10-12 |



**Letter from the applicant**

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**[001]** As skincare product specialists, we have intensively researched wrinkling of the skin. We have found new products and processes, which we consider innovative and for which we would like to apply for a patent.

**[002]** Facial wrinkles are a visible sign of ageing skin. There are various ways of reducing facial wrinkles. These include face creams, facelift surgery and the injection of botulinum proteins such as the well-known BOTOX®.

**[003]** The injection of the botulinum protein directly into the facial muscle is one of the most effective means of reducing wrinkles. However, the injection must be performed by a qualified person, usually a specialist doctor, and the patient may find the procedure painful. Also, the treatment usually has to be repeated every three to six months because the botulinum protein's effect wears off considerably over time.

**[004]** We wanted to know how the botulinum protein could be applied to the face without medical assistance in such a way as to pass through the skin and reach the facial muscle. As you know, the skin is quite thick and prevents the body from dehydrating and harmful substances from passing into the body. Most compounds cannot pass through the skin to reach the muscle.

**[005]** We have now discovered that the botulinum protein can pass through the skin and directly reach the area where the wrinkles form if it is coupled to a polymer and/or is in the form of a nanoemulsion. For example, the botulinum protein can be coupled with polyethylene glycol (PEG). PEG can be obtained in the required purity from known producers.



**[006]** PEG usually has an average molecular weight of 200 to 35000 Dalton. The name given to polyethylene glycols is determined by their average molecular weight. PEG-5000, for example, has an average molecular weight of 5000 Dalton. The duration of botulinum protein's effect in the body can be extended significantly by coupling it to PEG.

**[007]** We have discovered that only botulinum protein-PEG conjugates containing medium-weight PEG have a longer-lasting effect in the body and are suitable for treating wrinkles. In the context of this application, we consider medium-weight PEG to be PEG with an average molecular weight of 2000 to 15000 Dalton, preferably 2000 to 10000 Dalton and especially preferably 5000 Dalton.

**[008]** We also coupled the botulinum protein to other polymers, namely polyvinylpyrrolidone or hyaluronic acid. When selecting the conjugates for wrinkle treatment, it is essential that, in addition to having a significant wrinkle-reduction effect, the conjugates used are skin-friendly and do not cause any inflammation, pustules or peeling of the skin or trigger allergies. A slight redness which disappears by itself within a few hours after the application is, however, acceptable.

**[009]** A suitable nanoemulsion can be obtained either with the botulinum protein alone or with a botulinum protein-polymer conjugate. In nanoemulsions the emulsion droplets have an average droplet diameter of less than 1000 nm. Owing to their small average droplet diameter, they differ distinctly from conventional emulsions, which have average droplet diameters over 2000 nm.

**[010]** The nanoemulsions can be produced according to known methods. The droplets in the nanoemulsion have an average diameter of at most 1000 nm, preferably less than 500 nm or less than 200 nm, and especially preferably approximately 100 nm.



**[011]** For a stable effect, it is advantageous if the droplets in the nanoemulsion are all the same size. It is also advantageous if there are no large droplets, e.g. with a diameter of more than 500 nm, in the nanoemulsion.

**[012]** The skilled person knows that in a process for making nanoemulsions at least two immiscible phases are used, namely an aqueous and an oil phase. In our case, the aqueous phase can be, for example, water, a salt solution or an ethanol-water-mixture. Examples of oil phases are palm oil, almond oil, sunflower oil, soy oil, olive oil, silicon oil, avocado oil, or any other oil suitable for cosmetic use. The oil phase can also contain surfactants such as lecithin, phospholipids, polysorbate or stearylamine, lecithin being preferred because it is well tolerated. The botulinum protein or its conjugate are added to the aqueous phase.

**[013]** To produce nanoemulsions, high shear forces must be generated. For this, known methods such as, for instance, high pressure homogenisation are used. In our firm, we use a microfluidiser as high pressure homogeniser. Obtaining a nanoemulsion by our method requires that the two phases are mixed and exposed to a pressure of at least 1000 bar for a period of at least 30 seconds to at most 10 minutes. It has proved advantageous to expose the two mixed phases to a pressure of 1500 to 2000 bar to obtain droplets having an average diameter of less than 500 nm.

**[014]** When producing nanoemulsions, we noticed that the composition of the phases used for the method has an effect on the droplet size distribution. Two phases are mixed, the aqueous phase making up at least 90 vol.% and the oil phase 10 vol.% or less. The weight ratio of surfactant to oil is 2:1 or more, e.g. 2.5:1 or 3:1. If the mixed phases are now exposed to the advantageous high pressures mentioned above, droplets of the same size with a diameter of 100 nm +/- 10 nm are obtained.



**[015]** The botulinum protein conjugates and/or the nanoemulsion are stirred into a commercially available cream. This botulinum-protein-containing cream is best applied to the desired part of the face using a pipette in order to achieve a delivery of cream to the skin's surface as targeted as possible. Suitable pipettes are, for example, disposable pipettes made from polyethylene with a nominal volume of 1 to 10 ml and integrated suction bellows. Such disposable pipettes are sold by our subsidiary Easycare, Inc. under the brand name CANULETTA. Suitable pipettes can be sold together with our cream as a kit.

**[016]** All creams known to the skilled person can be used to produce the botulinum-protein-containing cream. Other additives can be added to this cream, such as, for example, thickeners, colouring agents, fragrances, preservatives, or other substances which have the effect of smoothing out wrinkles such as vitamin C, retinol, collagen or coenzyme Q.

**[017]** The botulinum-protein-containing cream is generally applied every 1 to 4 months. If the wrinkling is moderate, it is recommended that it be applied at two-monthly intervals. In cases of heavy wrinkling, the cream can also be applied monthly.

The following examples illustrate our invention.

#### **[018] Example 1**

##### Production of the botulinum protein conjugate

7.5 mg PEG-5000 or PEG-10000 is added to 1.5 mg botulinum protein and stirred over night at room temperature. We have produced conjugates of botulinum protein with polyvinylpyrrolidone or hyaluronic acid by analogous methods.

#### **[019] Example 2**

##### Production of a botulinum-protein-containing cream

5 mg of one of the botulinum protein conjugates from example 1 or 1 mg of unconjugated botulinum protein is stirred into 350 g of a commercially available cream.



**[020] Example 3**Production of a nanoemulsion cream

To produce a nanoemulsion, 250 g lecithin is mixed with 100 g almond oil in a weight ratio of 2.5:1 and then heated to 40°C. 5 mg of one of the botulinum protein-polymer conjugates from example 1 or 1 mg of unconjugated botulinum protein are dissolved in 100 ml of distilled water. This aqueous solution is added to the mixture of almond oil and lecithin. After stirring, the mixture is exposed to a pressure of 1500 bar in a microfluidiser for 2 minutes. The nanoemulsion so obtained is mixed with a commercially available cream in a ratio of 1:1. Table 1 shows the average diameter of the droplets, as measured by dynamic light scattering.

**Table 1**

Composition	Weight ratio Lecithin: almond oil	Average droplet diameter [nm]
Botulinum protein (unconjugated)	1:1	550
Botulinum protein (unconjugated)	2.5:1	110
Botulinum protein-PEG-5000 conjugate	1:1	500
Botulinum protein-PEG-5000 conjugate	2.5:1	90

**[021] Example 4**

The botulinum-protein-containing cream from example 2 or the botulinum-protein-containing nanoemulsion cream from example 3 was applied to the foreheads of five test persons aged 50 to 60 with distinct forehead wrinkling. Using a pipette, 0.5 to 0.8 ml of each cream was applied to a test person's forehead and massaged into the skin until it was no longer visible. The forehead was then observed one day later and, thereafter, weekly for a period of 4 months and compared by means of photographs and video images. The reduction in wrinkles after 2 weeks/4 weeks/8 weeks/12 weeks and any side effects are set out in Table 2 for the botulinum-protein-containing cream and in Table 3 for the botulinum-protein-containing nanoemulsion cream.



**Table 2: Botulinum-protein-containing cream**

Composition	Polymer used	Wrinkle reduction in % after (2/4/8/12) weeks	Side effects
1	-	10/0/0/0	None
2	PEG-5000	35/35/30/25	Slight redness for 1 hour
3	PEG-10000	35/30/25/20	None
4	Polyvinylpyrrolidone	50/50/40/40	Severe pustules in the forehead area for 1 week
5	Hyaluronic acid	5/0/0/0	None

**Table 3: Botulinum-protein-containing nanoemulsion cream**

Composition	Polymer used	Wrinkle reduction in % after (2/4/8/12) weeks	Side effects
6	-	45/30/20/10	None
7	PEG-5000	60/55/50/35	None
8	PEG-10000	50/50/40/30	None
9	Polyvinylpyrrolidone	90/80/60/40	Peeling of forehead skin
10	Hyaluronic acid	15/10/0/0	None



The tests show that using the creams containing the botulinum protein-PEG conjugate leads to an acceptable level of wrinkle reduction with no or only very minor side effects. A level of wrinkle reduction below a percentage of 20 % is unacceptable.

**[022]** We would like to draw your attention to the two documents D1 and D2 in the annex.



**[023]** Since the competition in this field is very active, we would ask you to file the application promptly. We do not wish to pay any claim fees.

Yours sincerely,  
Beppo Dellaruga





## Document D1

### Specialist information for the cosmetic branch, December 2013

**[001]** Beauty-conscious consumers have known ESTATICA, a Brazilian company, and its face cream KENTORA for several years. This botulinum-protein-containing cream has proved to be an effective means of smoothing out fine facial wrinkles. However, when it comes to treating deeper wrinkles, there is no alternative to injecting botulinum protein.

**[002]** ESTATICA has therefore expanded its activities to the lucrative market for the treatment of deep wrinkles by injection. ESTATICA has now achieved a crucial breakthrough in wrinkle treatment: whereas wrinkles have up to now had to be removed by injecting botulinum protein every 3 to 6 months, this unpleasant procedure now only needs to be performed about once a year. The company's researchers have coupled the known botulinum protein with a polymer and so extended the duration of its effect.

**[003]** TEEN-KAR, the botulinum preparation presented now, comprises a conjugate of botulinum protein and a polyethylene glycol from the so-called "medium-weight" group of polyethylene glycols (average molecular weight of approximately 2500 to approximately 15000 Dalton). TEEN-KAR is offered as an aqueous solution.

**[004]** Tests on 50 users have confirmed that the preparation is skin-friendly and has no notable side effects after its injection.

**[005]** In most patients, the effect lasted for at least 10 months, a further treatment being recommended after 12 months. In isolated cases, the wrinkles only became significantly visible again 14 months after the injection. That is a success as yet unrivalled by any other producer of botulinum protein for injection. TEEN-KAR will come on to the market in the next few weeks.

**[006]** ESTATICA is in the process of expanding its range of products even further and enhancing the effectiveness of its botulinum preparations. To that end, it could also use other polymers in lieu of polyethylene glycol.



**[007]** Manuel da Silva, ESTATICA 's managing director, sees potential for development in how the product is applied: one possibility would be an application system allowing patients to remove their wrinkles themselves by administering their own injections. However, it will take some time yet for that to be achieved. The fact is that injections administered by a doctor still deliver the best results.

**[008]** Nanotechnology could perhaps also help in future to provide even better botulinum protein injections for treating wrinkles.



**Document D2**

**WO 2008/34445422**

**[001]** The present invention provides a method of producing nanoemulsions containing proteins for use in the cosmetic, pharmaceutical or food industries.

**[002]** Emulsions in which the emulsion droplets have an average droplet diameter of less than 1000 nm are called nanoemulsions. Owing to their small droplet diameter, they differ distinctly from conventional emulsions, which have average droplet diameters of 2000 to 5000 nm. Nanoemulsions are often more stable and/or effective.

**[003]** A variety of proteins can be used in the method, including insulin, growth factors, hormones, milk proteins such as casein, bacterial proteins such as botulinum protein or vegetable proteins.

**[004]** The invention pertains to a method of producing a nanoemulsion. In a preferred embodiment, a botulinum protein is used in the method. The invention further provides a method of cosmetic treatment of wrinkles wherein a nanoemulsion containing botulinum protein is applied to a person's face.

**[005]** The nanoemulsions are generally produced by mixing an aqueous phase and an oil phase and by exposing the mixture to high shear forces. The shear forces are generated by, for example, high pressure, cavitation and/or microfluidisation, the pressure used being 1000 bar or higher (between 1200 and 2000 bar and even higher in special cases). In one specific embodiment, the two phases undergo microfluidisation for less than 10 minutes, preferably for less than 7, 6, 5, 4 or 3 minutes. A period of 2 minutes or less is especially preferred.



**[006]** The nanoemulsions obtained in this way consist of droplets having a diameter of less than 1000 nm, 500 nm, 300 nm, 200 nm or 100 nm.

**[007]** The aqueous phase can be, for example, water, a salt solution, an ethanol-water-mixture, or glucose solution. Examples of oil phases are palm oil, almond oil, sunflower oil, soy oil, olive oil, avocado oil or any other oil, as well as vegetable oils modified with polyethylene glycol, provided they are suitable for cosmetic and pharmaceutical use. The two phases are mixed, the aqueous phase usually making up 70 vol.% to 99 vol.%, 80 vol.% to 99 vol.% or 90 vol.% to 99 vol.%. In one embodiment, the oil makes up 1 vol.% to 30 vol.%, preferably 1 vol.%, 2 vol.%, 3 vol.%, 4 vol.%, 5 vol.%, 6 vol.%, 7 vol.%, 8 vol.%, 9 vol.% or 10 vol.%.

**[008]** Surfactants such as lecithin, phospholipids, polysorbate or stearylamine can also be added to the oil phase, the ratio of surfactant to oil being between 1:1 and 1:5. In some embodiments, the ratio of surfactant to oil is 1:1, 1:2, 1:2.5 or 1:3.

#### **[009] Example 1**

This example shows an embodiment of a nanoemulsion produced by microfluidisation and containing a botulinum protein. 100 g polysorbate and 250 g soy oil were mixed and then heated to 40°C. 1 mg of botulinum protein was dissolved in 100 ml of distilled water. This aqueous solution was added to the mixture of soy oil and polysorbate. After stirring, the mixture was exposed to a pressure of 1500 bar in a microfluidiser for 2 minutes. The resulting nanoemulsion was analysed for its droplet size. The droplet diameters obtained were mainly 200 nm, 100 nm and 300 nm.



### **[010] Example 2**

This example shows that the protein in the nanoemulsion can pass through the skin and retains its activity. The nanoemulsion containing botulinum protein from example 1 was stirred into an equal amount of a commercially available cream. Using a pipette, 0.7 ml of this nanoemulsion cream was applied to the forehead of the female test person with distinct forehead wrinkling and massaged into the skin until the cream was no longer visible. The forehead was then observed one day later and, subsequently, after 1 week, 2 weeks, 4 weeks, 8 weeks and 12 weeks and compared using photographs and video images.

### **[011] Result**

Within one week, the treated facial muscle was paralysed and there was an 80% reduction in wrinkles. The effect began to wear off after 2 weeks and, after 4 weeks, the wrinkles had almost all reformed. After 8 weeks at the latest, there was no longer any visible effect. The test person did not complain of any side effects such as inflammation or redness during the tested 12-week period. It can be inferred from this experiment that the nanoemulsion applied to the skin showed a significant effect which, for a period of approximately 2 weeks, was comparable to that expected of a standard treatment by injection of botulinum protein.

### **Claims**

1. A method of producing a nanoemulsion, comprising the following steps:
  - (a) mixing of an oil phase and an aqueous phase containing protein and, optionally, a surfactant, and
  - (b) exposure of the mixture to a pressure of more than 1000 bar.
2. Method according to claim 1, wherein botulinum protein is used as the protein.
3. Method of cosmetic treatment of wrinkles wherein a nanoemulsion containing botulinum protein is applied to a person's face.

