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# Peptide fibrils as nanocarrier for targeted therapy

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## INTRODUCTION

The use of drug delivery systems, particularly nanocarriers, has gained prominence as a result of their improved safety and efficacy compared to conventional therapies. Nanodrugs, consisting of nanocarriers conjugated with cytostatic molecules, have the ability to target cancer cells through active and/or passive targeting mechanisms. Antibody-drug conjugates (ADCs) offer a less burdensome, more selective, and potentially more effective therapeutic option. Despite its efficacy, the affordability of ADCs is a challenging issue, limiting access for a significant number of patients. Current approaches include a variety of biologically derived agents, including antibodies, peptides, and aptamers, to target nanotherapeutics to specific cancer cell receptors, such as HER2 [1]. Peptide fibrils, which self-organise from proteins and/or peptides, have a large surface-area-to-volume ratio, which allows for higher drug loading and attachment of targeting molecules. Their physicochemical and mechanical properties can also be regulated by altering the amino acid sequence of the peptides that form the fibrils. We studied the kinetics of fibril growth using an innovative method that combined electrochemical impedance spectroscopy (EIS) with cyclic voltammetry (CV). We also used a thioflavin (ThT) assay. The morphology of the fibrils was visualised using transmission electron microscopy (TEM) and atomic force microscopy (AFM). Additionally, we compared the molecular size of the peptide fibrils on day '7' of fibrillization using dynamic light scattering (DLS).

## PEPTIDE DESIGN AND SYNTHESIS

One of the well-described fibril-forming peptides is the peptide with the sequence QAGIVV (FC). The amino acid sequence of the FC peptide is a fragment 55-60 of the human cystatin C (HCC) protein, whose sequence has been shuffled, the so-called shuffle peptide [2]. Our group conducted studies that confirm that its self-association ability does not disappear even after adding additional amino acid residues [3]. Two analogues were designed. The GFC peptide, which is the glycine analogue of the FC peptide, will serve as a carrier to which we will attach an antibody or affibody and a drug with known cytostatic activity. Additionally, we synthesized the CGFC peptide, which will enable drug attachment by forming a covalent bond.


***Figure 1.*** *Amino acid sequence of the CGFC/GFC/FC peptide.*

All of the peptides were synthesised on a solid support using Fmoc chemistry, and then purified using reversed-phase high-performance liquid chromatography and mass spectrometry. Then, the obtained peptide was fibrillized. For this purpose, the peptide was dissolved in phosphate buffered saline (PBS) at pH 7.4, C = 1 mg/ml and incubated for 7 days at 37°C with constant orbital shaking.

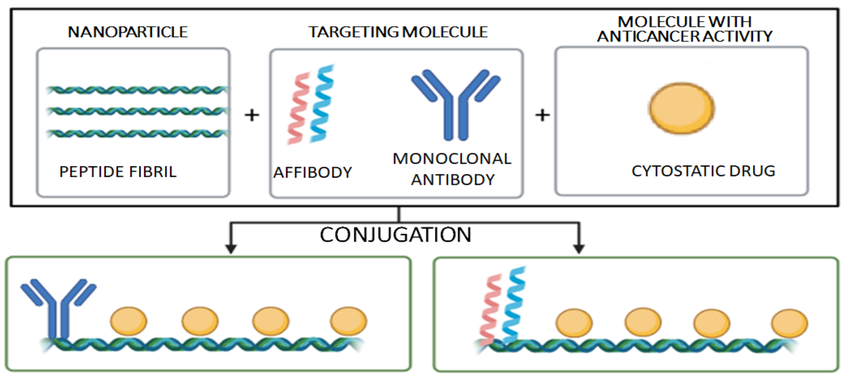



***Figure 2.*** *Conceptual illustration of nanodrugs formation.*

## METHODS AND RESULTS

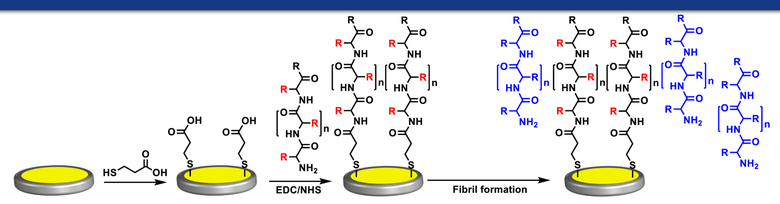
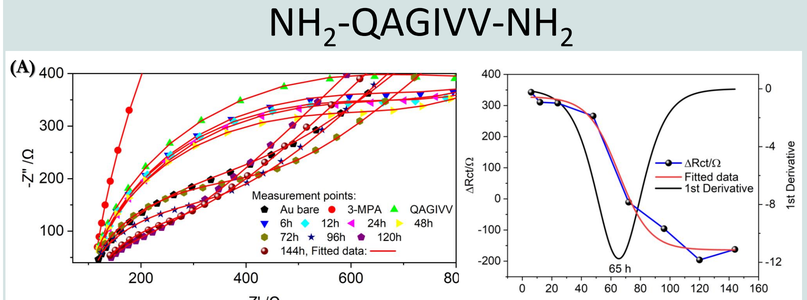



***Figure 3.*** *Scheme of gold electrode functionalization with the correct peptide in order to obtain sensitive electrode fibrils growth.*

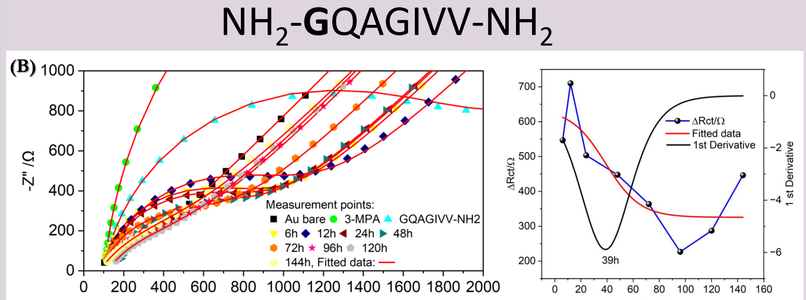
Each of the peptides was used for gold electrode functionalization purposes. The surface gold electrode was modified with mercaptocarboxylic acid to obtain carboxyl groups. This modification allows covalent attachment of the fibrilogenic peptide with the NH2 group in C-terminus (FC / GFC / CGFC) to the electrode surface (Figure 3). The preliminary of electrochemical impedance spectroscopy (EIS) spectra obtained show (Figure 4), that the electrode was functionalised successfully with the peptide. In the next step, the electrode was immersed in PBS buffer with the correct peptide (FC/GFC/CGFC) and incubated at 37°C using continuous shaking.

### ELECTROCHEMICAL MEASUREMENTS

#### FC
$NH_2$-QAGIVV-$NH_2$




#### GFC
$NH_2$-**G**QAGIVV-$NH_2$




#### CGFC
$NH_2$-**CG**QAGIVV-$NH_2$

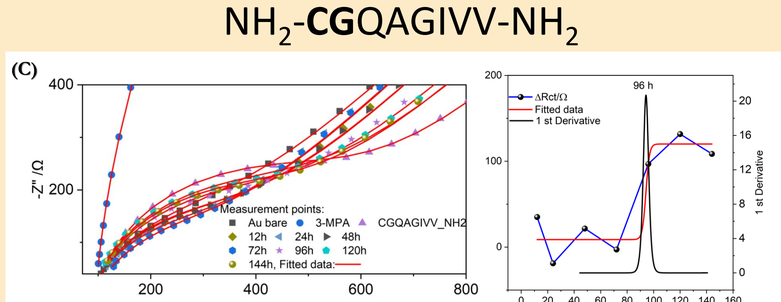



***Figure 4.*** *Electrochemical impedance spectroscopy spectra obtained for modified electrodes and after incubation in fibril growth solution recorded at different time points for* ***(A)*** *FC;* ***(B)*** *GFC;* ***(C)*** *CGFC; peptide.*

### TIOFLAVIN T ASSAY

Electrochemical impedance spectroscopy (EIS) spectra were obtained for modified electrodes before and after incubation in a fibril growth solution, recorded at different time points for the FC peptide. The electrode was immersed in a 1 mg/ml solution of the FC peptide in PBS buffer and incubated at 37°C with continuous shaking. The EIS spectra reveal that FC fibrils grew slowly during the first 20 hours, after which their growth rate increased rapidly, reaching a stable level after approximately 65 hours.

Electrochemical impedance spectroscopy (EIS) spectra were obtained for modified electrodes before and after incubation in a fibril growth solution, recorded at different time points for the FC peptide. The electrode was immersed in a 1 mg/ml solution of the FC peptide in PBS buffer and incubated at 37°C with continuous shaking. The EIS spectra reveal that GFC fibrils grew slow for the first 39 hours, after 80h, then their growth increased and after about 39 hours they reached a stable level.

Electrochemical impedance spectroscopy (EIS) spectra were obtained for modified electrodes before and after incubation in a fibril growth solution, recorded at different time points for the CGFC peptide. The electrode was immersed in a 1 mg/ml solution of the CGFC peptide in PBS buffer and incubated at 37°C with continuous shaking. The EIS spectra reveal that FC fibrils grew slowly during the first 75 hours, after which their growth rate increased rapidly, reaching a stable level after approximately 96 hours.

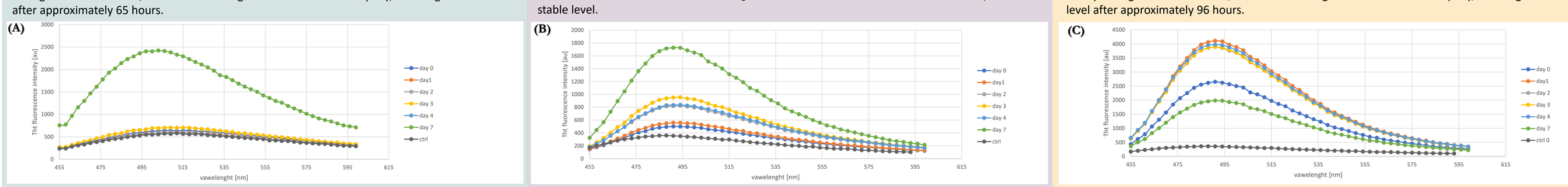



***Figure 5.*** *Graph of the dependence of the fluorescence intensity of the peptide-thioflavin T complex on incubation time relative to the control consisting of PBS solution for* ***(A)*** *FC;* ***(B)*** *GFC;* ***(C)*** *CGFC; peptide.*

### AFM / TEM

A 0.1 mg/ml FC peptide solution in PBS with thioflavin T (ThT), with a final peptide concentration of 10 µM, was used for the measurement. PBS with ThT served as the negative control. The sample was excited at 420 nm, and the emission spectrum was recorded in the range of 455 to 600 nm. The absorption maximum was observed at 482 nm. An increase in fluorescence intensity was observed over consecutive days. As shown in Figure 5 (A), the maximum fluorescence intensity was reached on day 7.

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A 0.1 mg/ml FC peptide solution in PBS with thioflavin T (ThT), with a final peptide concentration of 10 µM, was used for the measurement. PBS with ThT served as the negative control. The sample was excited at 420 nm, and the emission spectrum was recorded in the range of 455 to 600 nm. The absorption maximum was observed at 482 nm. An increase in fluorescence intensity was observed over consecutive days. As shown in Figure 5 (C), it can seen that the maximum of fluorescence intensity is at the first 3 days.

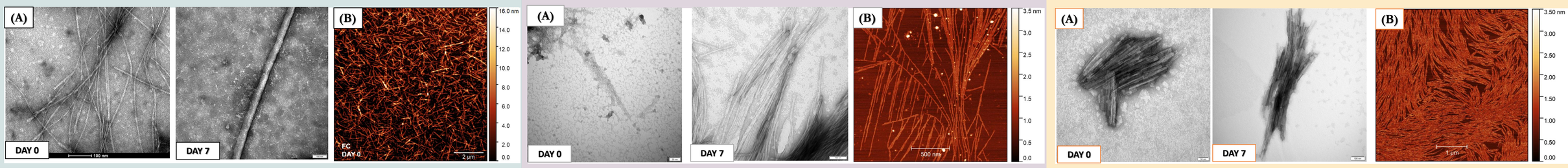


***Figure 6. (A)****Transmission electron micrographs (TEM) for peptides FC, GFC and CGFC taken before and on the 7rd day of incubation in PBS buffer;* ***(B)*** *Atomic force microscopy (AFM) images of fibrils taken briefly after dissolution in PBS buffer.*

### DLS

The FC peptide forms fibrils immediately after dissolving in PBS buffer. As observed in TEM images, the FC peptide forms very long fibers, exceeding 900 nm in length and approximately 10 nm in width. AFM images also reveal the formation of a dense, three-dimensional network of peptide fibrils.

The GFC peptide forms fibrils immediately after dissolving in PBS buffer. By the 7th day of incubation, the GFC peptide has developed a network of long, parallel fibrils. These fibrils are over 1 µm in length and approximately 5 nm in width, as seen in the images. The AFM image reveals a fibril arrangement resembling a horsetail pattern.

The CGFC peptide forms fibrils immediately after dissolving in PBS buffer. As seen in TEM images, the CGFC peptide forms fibers ranging from 100 to 500 nm in length and approximately 10 nm in width, which are tightly attached to one another. AFM images also show the formation of a dense, three-dimensional network of peptide fibrils.

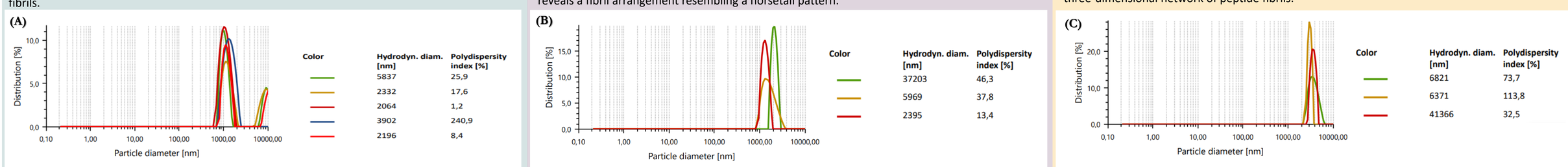


***Figure 7.*** *Particle size distribution (intensity) obtained for* ***(A)*** *FC;* ***(B)*** *GFC;* ***(C)*** *CGFC; peptide in day 7th of incubation in PBS.*

The FC peptide has a mean hydrodynamic diameter of 3444 nm. A polydispersity index of 35.8% indicates moderate uniformity in particle size. Two dominant size peaks at 5665 nm and 4392 nm suggest the presence of two distinct particle populations.

The GFC peptide has an average hydrodynamic diameter of 15,189 nm, indicating the presence of large aggregates. The polydispersity index is 32.5%, reflecting moderate particle size variability. The primary size peak is observed at 1712.4 nm, with a higher light transmission of 75.1%.

The CGFC peptide is characterized by a large hydrodynamic diameter of 18,186 nm. The high polydispersity index of 73.3% indicates a broad distribution of particle sizes. The primary size peak is observed at 3480 nm.

## CONCLUSION

**Our study shows that minor changes in the peptide sequence (e.g., the addition of an additional amino acid residue) have a significant effect on their fibrilization properties, as illustrated by the differences between the FC, GFC, and CGFC peptides. The FC peptide shows slow initial fibril growth, as evidenced by ThT measurements that correlate with the EIS results. The FC formed very long fibrils, as observed by microscopy. The GFC peptide also reaches a peak in ThT fluorescence after 7 days, but are stabilized earlier (after 39 hours) and forms longer and narrower fibrils. The CGFC peptide, because of its ability to form dimers via disulphide bonds (owing to cysteine residues), resulted in significantly larger fibrils, as confirmed by DLS measurements showing a large hydrodynamic diameter. The ThT assay showed that the CGFC peptide stabilized its fibril growth after 96 hours, as also observed by EIS measurements. The combination of AFM/TEM, ThT assay, and EIS provided a comprehensive understanding of the fibril formation process, revealing differences in the kinetics, morphology, and structural properties of the peptides.**

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